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(54) Title: IMPROVED PROCESS OF PREPARING MRNA-LOADED LIPID NANOPARTICLES

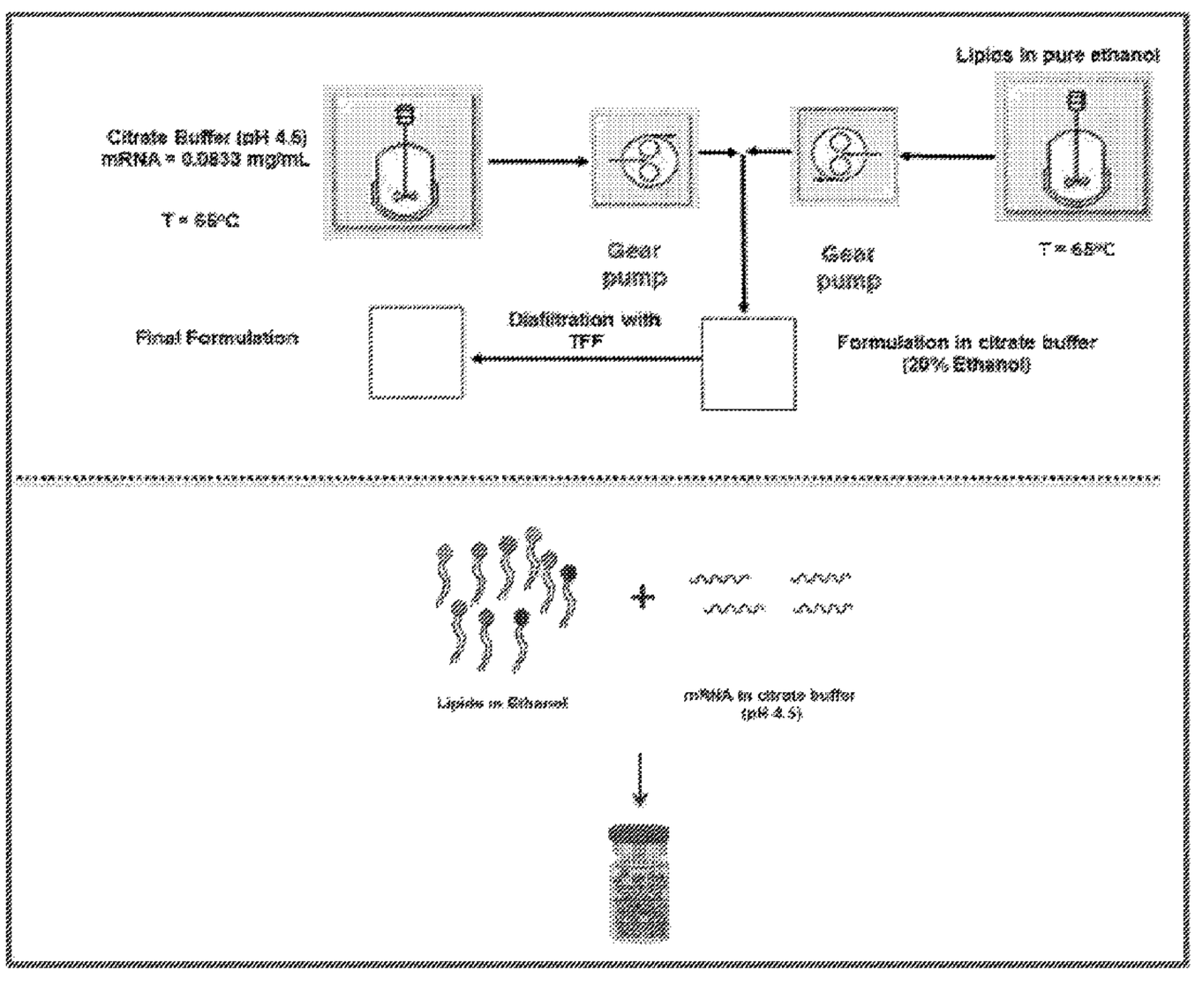


FIG. 1

(57) Abrégé/Abstract:
The present invention provides an improved process for lipid nanoparticle formulation and mRNA encapsulation. In some embodiments, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising a step of mixing a solution of pre-formed lipid nanoparticles and mRNA.

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(54) Title: IMPROVED PROCESS OF PREPARING MRNA-LOADED LIPID NANOPARTICLES

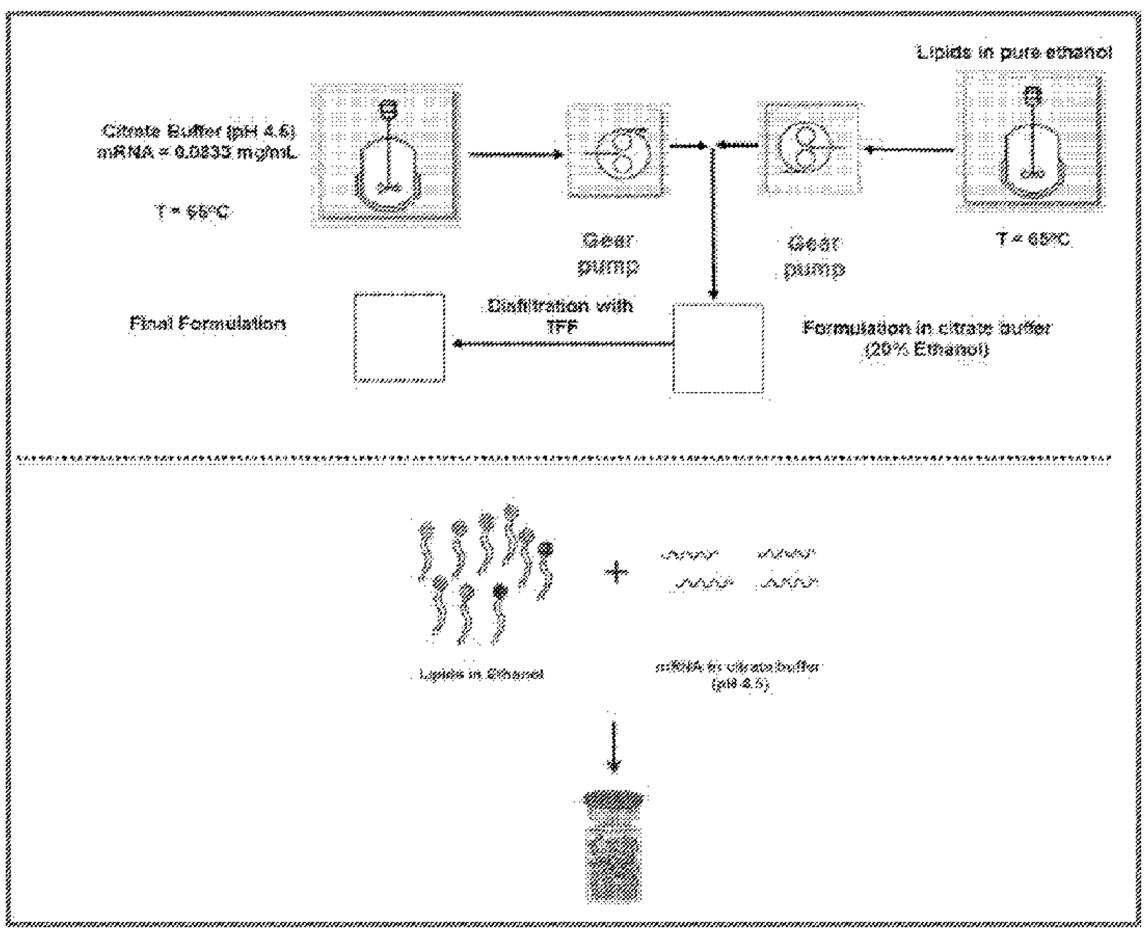


FIG. 1

(57) Abstract: The present invention provides an improved process for lipid nanoparticle formulation and mRNA encapsulation. In some embodiments, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising a step of mixing a solution of pre-formed lipid nanoparticles and mRNA.

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IMPROVED PROCESS OF PREPARING MRNA-LOADED LIPID NANOPARTICLES

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 62/420,413, filed November 10, 2016 and U.S. Provisional Application Serial No. 62/580,155, filed November 1, 2017, the disclosures of which are hereby incorporated by reference.

SEQUENCE LISTING

[0002] The present specification makes reference to a Sequence Listing (submitted electronically as a .txt file named "MRT-1246WO_SL" on November 10, 2017). The .txt file was generated November 10, 2017 and is 17,482 bytes in size. The entire contents of the Sequence Listing are herein incorporated by reference.

BACKGROUND

[0003] Messenger RNA therapy (MRT) is becoming an increasingly important approach for the treatment of a variety of diseases. MRT involves administration of messenger RNA (mRNA) to a patient in need of the therapy for production of the protein encoded by the mRNA within the patient's body. Lipid nanoparticles are commonly used to encapsulate mRNA for efficient *in vivo* delivery of mRNA.

[0004] To improve lipid nanoparticle delivery, much effort has focused on identifying novel lipids or particular lipid compositions that can affect intracellular delivery and/or expression of mRNA, e.g., in various types of mammalian tissue, organs and/or cells (e.g., mammalian liver cells). However, these existing approaches are costly, time consuming and unpredictable.

SUMMARY OF INVENTION

[0005] The present invention provides, among other things, an improved process for preparing mRNA-loaded lipid nanoparticles. In particular, the present invention is based on the surprising discovery that encapsulating mRNA by combining pre-formed lipid nanoparticles with mRNA results in formulated particles that exhibit unexpectedly efficient *in vivo* delivery of the mRNA and surprisingly potent expression of protein(s) and/or peptide(s) that the mRNA encodes.

[0006] As compared to conventional processes, the inventive process described herein provides higher potency and better efficacy of lipid nanoparticle delivered mRNA, thereby shifting the therapeutic index in a positive direction and providing additional advantages, such as lower cost, better patient compliance, and more patient friendly dosing regimens. mRNA-loaded lipid nanoparticle formulations provided by the present invention may be successfully delivered *in vivo* for more potent and efficacious protein expression via different routes of administration such as intravenous, intramuscular, intra-articular, intrathecal, inhalation (respiratory), subcutaneous, intravitreal, and ophthalmic.

[0007] This inventive process can be performed using a pump system and is therefore scalable, allowing for improved particle formation/formulation in amounts sufficient for, e.g., performance of clinical trials and/or commercial sale. Various pump systems may be used to practice the present invention including, but not limited to, pulse-less flow pumps, gear pumps, peristaltic pumps, and centrifugal pumps.

[0008] This inventive process also results in superior encapsulation efficiency, mRNA recovery rate, and homogeneous particle sizes.

[0009] Thus, in one aspect, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising a step of mixing a solution comprising pre-formed lipid nanoparticles and a solution comprising mRNA such that lipid nanoparticles encapsulating mRNA are formed. As used herein, pre-formed lipid nanoparticles are substantially free of mRNA. In some embodiments, preformed lipid nanoparticles are referred to as empty lipid nanoparticles.

[0010] In some embodiments, the process according to the present invention includes a step of heating one or more of the solutions (i.e., applying heat from a heat source to the solution) to a temperature (or to maintain at a temperature) greater than ambient temperature, the one more solutions being the solution comprising the pre-formed lipid nanoparticles, the solution comprising the mRNA and the mixed solution comprising the lipid nanoparticle encapsulated mRNA. In some embodiments, the process includes the step of heating one or both of the mRNA solution and the pre-formed lipid nanoparticle solution, prior to the mixing step. In some embodiments, the process includes heating one or more one or more of the solution comprising the pre-formed lipid nanoparticles, the solution comprising the mRNA and the solution comprising the lipid nanoparticle encapsulated mRNA, during the mixing step. In some embodiments, the process includes the step of heating the lipid nanoparticle encapsulated mRNA, after the mixing step. In some embodiments, the temperature to which one or more of the solutions is heated (or at which one or more of the solutions is maintained) is or is greater than about 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, or 70 °C. In some embodiments, the temperature to which one or more of the solutions is heated ranges from about 25-70 °C, about 30-70 °C, about 35-70 °C, about 40-70 °C, about 45-70 °C, about 50-70 °C, or about 60-70 °C. In some embodiments, the temperature greater than ambient temperature to which one or more of the solutions is heated is about 65 °C.

[0011] In some embodiments, the process according to the present invention includes maintaining at ambient temperature (i.e., not applying heat from a heat source to the solution) one or more of the solution comprising the pre-formed lipid nanoparticles, the solution comprising the mRNA and the mixed solution comprising the lipid nanoparticle encapsulated mRNA. In some embodiments, the process includes the step of maintaining at ambient temperature one or both of the mRNA solution and the pre-formed lipid nanoparticle solution, prior to the mixing step. In some embodiments, the process includes maintaining at ambient temperature one or more one or more of the solution comprising the pre-formed lipid nanoparticles, the solution comprising the mRNA and the solution comprising the lipid nanoparticle encapsulated mRNA, during the mixing step. In some embodiments, the process includes the step of maintaining at ambient temperature the lipid nanoparticle encapsulated

mRNA, after the mixing step. In some embodiments, the ambient temperature at which one or more of the solutions is maintained is or is less than about 35 °C, 30 °C, 25 °C, 20 °C, or 16 °C. In some embodiments, the ambient temperature at which one or more of the solutions is maintained ranges from about 15-35 °C, about 15-30 °C, about 15-25 °C, about 15-20 °C, about 20-35 °C, about 25-35 °C, about 30-35 °C, about 20-30 °C, about 25-30 °C or about 20-25 °C. In some embodiments, the ambient temperature at which one or more of the solutions is maintained is 20-25 °C.

[0012] In some embodiments, the process according to the present invention includes performing at ambient temperature the step of mixing the solution comprising pre-formed lipid nanoparticles and the solution comprising mRNA to form lipid nanoparticles encapsulating mRNA.

[0013] In some embodiments, the pre-formed lipid nanoparticles are formed by mixing lipids dissolved in ethanol with an aqueous solution. In some embodiments, the lipids contain one or more cationic lipids, one or more helper lipids, and one or more PEG lipids. In some embodiments, the lipids also contain one or more cholesterol lipids. The pre-formed lipid nanoparticles are formed by the mixing of those lipids. Accordingly, in some embodiments, the pre-formed lipid nanoparticles comprise one or more cationic lipids, one or more helper lipids, and one or more PEG lipids. In some embodiments, the pre-formed lipid nanoparticles also contain one or more cholesterol lipids.

[0014] In some embodiments, the one or more cationic lipids are selected from the group consisting of cKK-E12, OF-02, C12-200, MC3, DLinDMA, DLinkC2DMA, ICE (Imidazol-based), HGT5000, HGT5001, HGT4003, DODAC, DDAB, DMRIE, DOSPA, DOGS, DODAP, DODMA and DMDMA, DODAC, DLenDMA, DMRIE, CLinDMA, CpLinDMA, DMOBA, DOcarbDAP, DLinDAP, DLincarbDAP, DLinCDAP, KLin-K-DMA, DLin-K-XTC2-DMA, 3-(4-(bis(2-hydroxydodecyl)amino)butyl)-6-(4-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)butyl)-1,4-dioxane-2,5-dione (Target 23), 3-(5-(bis(2-hydroxydodecyl)amino)pentan-2-yl)-6-(5-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)pentan-2-yl)-1,4-dioxane-2,5-dione (Target 24), N1GL, N2GL, V1GL and combinations thereof.

[0015] In some embodiments, the one or more cationic lipids are amino lipids. Amino lipids suitable for use in the invention include those described in WO2017180917, which is hereby incorporated by reference. Exemplary aminolipids in WO2017180917 include those described at paragraph [0744] such as DLin-MC3-DMA (MC3), (13Z,16Z)-N,N-dimethyl-3-nonyldocosa-13,16-dien-1-amine (L608), and Compound 18. Other amino lipids include Compound 2, Compound 23, Compound 27, Compound 10, and Compound 20. Further amino lipids suitable for use in the invention include those described in WO2017112865, which is hereby incorporated by reference. Exemplary amino lipids in WO2017112865 include a compound according to one of formulae (I), (Ial)-(Ia6), (lb), (II), (Ila), (III), (Ilia), (IV), (17-1), (19-1), (19-11), and (20-1), and compounds of paragraphs [00185], [00201], [0276]. In some embodiments, cationic lipids suitable for use in the invention include those described in WO2016118725, which is hereby incorporated by reference. Exemplary cationic lipids in WO2016118725 include those such as KL22 and KL25. In some embodiments, cationic lipids suitable for use in the invention include those described in WO2016118724, which is hereby incorporated by reference. Exemplary cationic lipids in WO2016118725 include those such as KL10, 1 ,2-dilinoleyloxy-N,N-dimethylaminopropane (DLin-DMA), and KL25.

[0016] In some embodiments, the one or more non-cationic lipids are selected from DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleyl-sn-glycero-3-phosphoethanolamine), DOPC (1,2-dioleyl-sn-glycero-3-phosphotidylcholine) DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), DOPG (1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)).

[0017] In some embodiments, the one or more PEG-modified lipids comprise a poly(ethylene) glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length.

[0018] In some embodiments, the pre-formed lipid nanoparticles are purified by a Tangential Flow Filtration (TFF) process. In some embodiments, greater than about 50%,

55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the purified nanoparticles have a size less than about 150 nm (e.g., less than about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, or about 50 nm). In some embodiments, substantially all of the purified nanoparticles have a size less than 150 nm (e.g., less than about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, or about 50 nm). In some embodiments, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified nanoparticles have a size ranging from 50-150 nm. In some embodiments, substantially all of the purified nanoparticles have a size ranging from 50-150 nm. In some embodiments, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified nanoparticles have a size ranging from 80-150 nm. In some embodiments, substantially all of the purified nanoparticles have a size ranging from 80-150 nm.

[0019] In some embodiments, a process according to the present invention results in an encapsulation rate of greater than about 90%, 95%, 96%, 97%, 98%, or 99%. In some embodiments, a process according to the present invention results in greater than about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% recovery of mRNA.

[0020] In some embodiments, the pre-formed lipid nanoparticles and mRNA are mixed using a pump system. In some embodiments, the pump system comprises a pulse-less flow pump. In some embodiments, the pump system is a gear pump. In some embodiments, a suitable pump is a peristaltic pump. In some embodiments, a suitable pump is a centrifugal pump. In some embodiments, the process using a pump system is performed at large scale. For example, in some embodiments, the process includes using pumps as described herein to mix a solution of at least about 1 mg, 5 mg, 10 mg, 50 mg, 100 mg, 500 mg, or 1000 mg of mRNA with a solution of pre-formed lipid nanoparticles, to produce mRNA encapsulated in lipid nanoparticles. In some embodiments, the process of mixing mRNA with pre-formed

lipid nanoparticles provides a composition according to the present invention that contains at least about 1 mg, 5mg, 10 mg, 50 mg, 100 mg, 500 mg, or 1000 mg of encapsulated mRNA.

[0021] In some embodiments, the solution comprising pre-formed lipid nanoparticles is mixed at a flow rate ranging from about 25-75 ml/minute, about 75-200 ml/minute, about 200-350 ml/minute, about 350-500 ml/minute, about 500-650 ml/minute, about 650-850 ml/minute, or about 850-1000 ml/minute. In some embodiments, the solution comprising pre-formed lipid nanoparticles is mixed at a flow rate of about 50 ml/minute, about 100 ml/minute, about 150 ml/minute, about 200 ml/minute, about 250 ml/minute, about 300 ml/minute, about 350 ml/minute, about 400 ml/minute, about 450 ml/minute, about 500 ml/minute, about 550 ml/minute, about 600 ml/minute, about 650 ml/minute, about 700 ml/minute, about 750 ml/minute, about 800 ml/minute, about 850 ml/minute, about 900 ml/minute, about 950 ml/minute, or about 1000 ml/minute.

[0022] In some embodiments, the mRNA is mixed in a solution at a flow rate ranging from about 25-75 ml/minute, about 75-200 ml/minute, about 200-350 ml/minute, about 350-500 ml/minute, about 500-650 ml/minute, about 650-850 ml/minute, or about 850-1000 ml/minute. In some embodiments, the mRNA is mixed in a solution at a flow rate of about 50 ml/minute, about 100 ml/minute, about 150 ml/minute, about 200 ml/minute, about 250 ml/minute, about 300 ml/minute, about 350 ml/minute, about 400 ml/minute, about 450 ml/minute, about 500 ml/minute, about 550 ml/minute, about 600 ml/minute, about 650 ml/minute, about 700 ml/minute, about 750 ml/minute, about 800 ml/minute, about 850 ml/minute, about 900 ml/minute, about 950 ml/minute, or about 1000 ml/minute.

[0023] In some embodiments, a process according to the present invention includes a step of first generating pre-formed lipid nanoparticle solution by mixing a citrate buffer with lipids dissolved in ethanol.

[0024] In some embodiments, a process according to the present invention includes a step of first generating an mRNA solution by mixing a citrate buffer with an mRNA stock solution. In certain embodiments, a suitable citrate buffer contains about 10 mM citrate, about 150 mM NaCl, pH of about 4.5. In some embodiments, a suitable mRNA stock

solution contains the mRNA at a concentration at or greater than about 1 mg/ml, about 10 mg/ml, about 50 mg/ml, or about 100 mg/ml.

[0025] In some embodiments, the citrate buffer is mixed at a flow rate ranging between about 100-300 ml/minute, 300-600 ml/minute, 600-1200 ml/minute, 1200-2400 ml/minute, 2400-3600 ml/minute, 3600-4800 ml/minute, or 4800-6000 ml/minute. In some embodiments, the citrate buffer is mixed at a flow rate of about 220 ml/minute, about 600 ml/minute, about 1200 ml/minute, about 2400 ml/minute, about 3600 ml/minute, about 4800 ml/minute, or about 6000 ml/minute.

[0026] In some embodiments, the mRNA stock solution is mixed at a flow rate ranging between about 10-30 ml/minute, about 30-60 ml/minute, about 60-120 ml/minute, about 120-240 ml/minute, about 240-360 ml/minute, about 360-480 ml/minute, or about 480-600 ml/minute. In some embodiments, the mRNA stock solution is mixed at a flow rate of about 20 ml/minute, about 40 ml/minute, about 60 ml/minute, about 80 ml/minute, about 100 ml/minute, about 200 ml/minute, about 300 ml/minute, about 400 ml/minute, about 500 ml/minute, or about 600 ml/minute.

[0027] In some embodiments, the lipid nanoparticles encapsulating mRNA are prepared with the pre-formed lipid nanoparticles by mixing an aqueous solution containing the mRNA with an aqueous solution containing the pre-formed lipid nanoparticles. In some embodiments, the aqueous solution containing the mRNA and/or the aqueous solution containing the pre-formed lipid nanoparticles is an aqueous solution comprising pharmaceutically acceptable excipients, including, but not limited to, one or more of trehalose, sucrose, lactose, and mannitol.

[0028] In some embodiments, one or both of a non-aqueous solvent, such as ethanol, and citrate are absent (i.e., below detectable levels) from one or both of the solution containing the mRNA and the solution containing the pre-formed lipid nanoparticles during the mixing addition of the mRNA to the pre-formed lipid nanoparticles. In some embodiments, one or both of the solution containing the mRNA and the solution containing the pre-formed lipid nanoparticles are buffer exchanged to remove one or both of non-aqueous solvents, such as ethanol, and citrate prior to the mixing addition of the mRNA to the

pre-formed lipid nanoparticles. In some embodiments, one or both of the solution containing the mRNA and the solution containing the pre-formed lipid nanoparticles include only residual citrate during the mixing addition of mRNA to the pre-formed lipid nanoparticles. In some embodiments, one or both of the solution containing the mRNA and the solution containing the pre-formed lipid nanoparticles include only residual non-aqueous solvent, such as ethanol. In some embodiments, one or both of the solution containing the mRNA and the solution containing the pre-formed lipid nanoparticles contains less than about 10mM (e.g., less than about 9mM, about 8mM, about 7mM, about 6mM, about 5mM, about 4mM, about 3mM, about 2mM, or about1mM) of citrate present during the addition of mRNA to the pre-formed lipid nanoparticles. In some embodiments, one or both of the solution containing the mRNA and the solution containing the pre-formed lipid nanoparticles contains less than about 25% (e.g., less than about 20%, about 15%, about 10%, about 5%, about 4%, about 3%, about 2%, or about 1%) of non-aqueous solvents, such as ethanol, present during the addition of mRNA to the pre-formed lipid nanoparticles. In some embodiments, the solution comprising the lipid nanoparticles encapsulating mRNA does not require any further downstream processing (e.g., buffer exchange and/or further purification steps) after the pre-formed lipid nanoparticles and mRNA are mixed to form that solution.

[0029] In another aspect, the present invention provides a composition of lipid nanoparticles encapsulating mRNA generated by a process described herein. In some embodiments, a substantial amount of the lipid nanoparticles are pre-formed. In some embodiments, at least 85% (e.g., at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) of the lipid nanoparticles are pre-formed. In some embodiments, the present invention provides a composition comprising purified lipid nanoparticles, wherein greater than about 90% of the purified lipid nanoparticles have an individual particle size of less than about 150 nm (e.g., less than about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, or about 50 nm) and greater than about 70% of the purified lipid nanoparticles encapsulate an mRNA within each individual particle. In some embodiments, greater than about 95%, 96%, 97%, 98%, or 99% of the

purified lipid nanoparticles have an individual particle size of less than about 150 nm (e.g., less than about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, or about 50 nm). In some embodiments, substantially all of the purified lipid nanoparticles have an individual particle size of less than about 150 nm (e.g., less than about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, or about 50 nm). In some embodiments, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified nanoparticles have a size ranging from 50-150 nm. In some embodiments, substantially all of the purified nanoparticles have a size ranging from 50-150 nm. In some embodiments, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified nanoparticles have a size ranging from 80-150 nm. In some embodiments, substantially all of the purified nanoparticles have a size ranging from 80-150 nm.

[0030] In some embodiments, greater than about 90%, 95%, 96%, 97%, 98%, or 99% of the purified lipid nanoparticles encapsulate an mRNA within each individual particle. In some embodiments, substantially all of the purified lipid nanoparticles encapsulate an mRNA within each individual particle. In some embodiments, a composition according to the present invention contains at least about 1 mg, 5 mg, 10 mg, 100 mg, 500 mg, or 1000 mg of encapsulated mRNA.

[0031] In some embodiments, a pre-formed lipid nanoparticle comprises one or more cationic lipids, one or more helper lipids and one or more PEG lipids. In some embodiments, each individual lipid nanoparticle also comprises one or more cholesterol based lipids. In some embodiments, the one or more cationic lipids are selected from the group consisting of cKK-E12, OF-02, C12-200, MC3, DLinDMA, DLinkC2DMA, ICE (Imidazol-based), HGT5000, HGT5001, HGT4003, DODAC, DDAB, DMRIE, DOSPA, DOGS, DODAP, DODMA and DMDMA, DODAC, DLenDMA, DMRIE, CLinDMA, CpLinDMA, DMOBA, DOcarbDAP, DLinDAP, DLincarbDAP, DLinCDAP, KLin-K-DMA, DLin-K-XTC2-DMA,

3-(4-(bis(2-hydroxydodecyl)amino)butyl)-6-(4-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)butyl)-1,4-dioxane-2,5-dione (Target 23), 3-(5-(bis(2-hydroxydodecyl)amino)pentan-2-yl)-6-(5-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)pentan-2-yl)-1,4-dioxane-2,5-dione (Target 24), N1GL, N2GL, V1GL and combinations thereof.

[0032] In some embodiments, the one or more cationic lipids are amino lipids. Amino lipids suitable for use in the invention include those described in WO2017180917, which is hereby incorporated by reference. Exemplary aminolipids in WO2017180917 include those described at paragraph [0744] such as DLin-MC3-DMA (MC3), (13Z,16Z)-N,N-dimethyl-3-nonyldocosa-13,16-dien-1-amine (L608), and Compound 18. Other amino lipids include Compound 2, Compound 23, Compound 27, Compound 10, and Compound 20. Further amino lipids suitable for use in the invention include those described in WO2017112865, which is hereby incorporated by reference. Exemplary amino lipids in WO2017112865 include a compound according to one of formulae (I), (Ial)-(Ia6), (lb), (II), (Ila), (III), (Ilia), (IV), (17-1), (19-1), (19-11), and (20-1), and compounds of paragraphs [00185], [00201], [0276]. In some embodiments, cationic lipids suitable for use in the invention include those described in WO2016118725, which is hereby incorporated by reference. Exemplary cationic lipids in WO2016118725 include those such as KL22 and KL25. In some embodiments, cationic lipids suitable for use in the invention include those described in WO2016118724, which is hereby incorporated by reference. Exemplary cationic lipids in WO2016118725 include those such as KL10, 1 ,2-dilinoleyloxy-N,N-dimethylaminopropane (DLin-DMA), and KL25.

[0033] In some embodiments, the one or more non-cationic lipids are selected from DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleyl-sn-glycero-3-phosphoethanolamine), DOPC (1,2-dioleyl-sn-glycero-3-phosphotidylcholine) DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), DOPG (1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)).

[0034] In some embodiments, the one or more cholesterol-based lipids is cholesterol or PEGylated cholesterol. In some embodiments, the one or more PEG-modified lipids

contain a poly(ethylene) glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length.

[0035] In some embodiments, the present invention is used to encapsulate mRNA containing one or more modified nucleotides. In some embodiments, one or more nucleotides is modified to a pseudouridine. In some embodiments, one or more nucleotides is modified to a 5-methylcytidine. In some embodiments, the present invention is used to encapsulate mRNA that is unmodified.

[0036] In yet another aspect, the present invention provides a method of delivering mRNA for *in vivo* protein production comprising administering into a subject a composition of lipid nanoparticles encapsulating mRNA generated by the process described herein, wherein the mRNA encodes one or more protein(s) or peptide(s) of interest.

[0037] In another aspect, the present invention provides a method for encapsulating messenger RNA (mRNA) in lipid nanoparticles wherein the method is performed without use of ethanol. In some embodiments, the method comprises a step of mixing a solution comprising one or more cationic lipids, one or more non-cationic lipids and one or more PEG-modified lipids with a solution comprising mRNA. In some embodiments, in the solution comprising the one or more cationic lipids, one or more non-cationic lipids and one or more PEG-modified lipids, at least a portion of the one or more cationic lipids, one or more non-cationic lipids and one or more PEG-modified lipids are present as pre-formed lipid nanoparticles. In some embodiments, the method is performed also without the use of citrate.

[0038] In certain embodiments, the method is performed without the use of any non-aqueous solvent. In some embodiments, there is no detectable ethanol and/or no detectable non-aqueous solvent. In some embodiments, there is no detectable citrate. In some embodiments, there is only a residual amount of ethanol and/or non-aqueous solvent that is present at less than about 25% (e.g., less than about 20%, about 15%, about 10%, about 5%, about 4%, about 3%, about 2%, or about 1%) of the solution. In some embodiments, there is only a residual amount of citrate that is less than 10mM (e.g., less than about 9mM, about

8mM, about 7mM, about 6mM, about 5mM, about 4mM, about 3mM, about 2mM, or about1mM).

[0039] In this application, the use of "or" means "and/or" unless stated otherwise. As used in this disclosure, the term "comprise" and variations of the term, such as "comprising" and "comprises," are not intended to exclude other additives, components, integers or steps. As used in this application, the terms "about" and "approximately" are used as equivalents. Both terms are meant to cover any normal fluctuations appreciated by one of ordinary skill in the relevant art.

[0040] Other features, objects, and advantages of the present invention are apparent in the detailed description, drawings and claims that follow. It should be understood, however, that the detailed description, the drawings, and the claims, while indicating embodiments of the present invention, are given by way of illustration only, not limitation. Various changes and modifications within the scope of the invention will become apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

[0041] The drawings are for illustration purposes only and not for limitation.

[0042] **Figure 1** shows a schematic of an exemplary lipid nanoparticle mRNA encapsulation process (Process A) that involves mixing lipids dissolved in ethanol with mRNA dissolved in an aqueous buffer, using a pump system.

[0043] **Figure 2** shows a schematic of an exemplary lipid nanoparticle mRNA encapsulation process (Process B) that involves mixing pre-formed empty lipid nanoparticles with mRNA dissolved in an aqueous buffer, using a pump system.

[0044] **Figure 3** depicts exemplary activity of expressed human ornithine transcarbamylase (hOTC) protein (in terms of citrulline production) in livers of OTC spf^{ash} mice 24 hours after a single 0.5 mg/kg dose of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or Process B. Before use, lipid nanoparticle

formulations made by Process A and Process B were stored in the frozen form at -80°C for (i) T =0 months (fresh without freezing), or (ii) T= 2.5 months.

[0045] **Figure 4** depicts exemplary activity of expressed hOTC protein (in terms of citrulline production) in livers of female OTC spf^{ash} mice 24 hours after a single 0.5 mg/kg dose of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or by Process B using different pump combinations. Lipid nanoparticle formulations made by Process B were prepared (1) using gear pumps, (2) using peristaltic pumps, (3) using peristaltic pumps at lower flow rates, and (4) using peristaltic pumps at different flow rates of mRNA and empty pre-formed lipid nanoparticles.

[0046] **Figure 5** depicts exemplary human argininosuccinate synthetase (ASS1) protein expression in 293T cells 16 hours post-transfection with either naked hASS1 mRNA (with lipofectamine) or hASS1 mRNA-encapsulated lipid nanoparticles (without lipofectamine) produced by Process A or Process B.

[0047] **Figure 6** shows exemplary immunohistochemical detection of human cystic fibrosis transmembrane conductance receptor (hCFTR) protein in rat lungs 24 hours after nebulization of hCFTR mRNA lipid nanoparticles prepared by Process B using different cationic lipids. Protein was detected in both the bronchial epithelial cells as well as the alveolar regions. Positive (brown) staining was observed in all mRNA lipid nanoparticles test article groups, as compared to saline-treated control rat lungs.

[0048] **Figure** 7 shows exemplary immunohistochemical detection of hCFTR protein in mouse lungs 24 hours after nebulization of hCFTR mRNA lipid nanoparticles prepared by Process B. Protein was detected in both the bronchial epithelial cells as well as the alveolar regions. Positive (brown) staining was observed for the mRNA lipid nanoparticle test article group, as compared to saline-treated control mice lungs.

[0049] **Figure 8** shows exemplary bioluminescent imaging of wild type mice 24 hours after intravitreal administration of Firefly Luciferase (FFL) mRNA encapsulated in lipid nanoparticles prepared by Process B.

[0050] **Figure 9** shows exemplary bioluminescent imaging of wild type mice 24 hours after topical application of eye drops containing FFL mRNA-encapsulated lipid nanoparticles formulated with polyvinyl alcohol and prepared by Process B.

[0051] **Figure 10** depicts exemplary serum phenylalanine levels in phenylalanine hydroxylase (PAH) knockout (KO) mice pre- and post-treatment of human PAH (hPAH) mRNA encapsulated in lipid nanoparticles prepared by Process B. Serum samples were measured 24 hours after a single subcutaneous administration.

[0052] **Figure 11** depicts exemplary activity of expressed hOTC protein (in terms of citrulline production) in the livers of OTC KO *spf*^{ash} mice 24 hours after a single subcutaneous administration of hOTC mRNA-encapsulated lipid nanoparticles prepared by Process B.

[0053] **Figure 12** depicts exemplary human ASS1 protein levels measured in the livers of ASS1 KO mice 24 hours after a single subcutaneous administration of hASS1 mRNA-encapsulated lipid nanoparticles prepared by Process B.

[0054] **Figure 13** depicts exemplary human erythropoietin (hEPO) protein levels measured in serum of treated mice at 6 hours and 24 hours after a single administration of different doses of hEPO mRNA-encapsulated lipid nanoparticles prepared by Process B. The routes of administration used were intradermal, subcutaneous and intramuscular delivery.

[0055] **Figure 14** shows a comparison of hEPO protein levels measured in the serum of treated mice 6 hours and 24 hours after a single intradermal dose of hEPO mRNA encapsulated in a lipid nanoparticle formulation made by Process A or by Process B.

[0056] **Figure 15** depicts a comparison of hEPO protein levels measured in the serum of treated mice 6 hours and 24 hours after a single intramuscular dose of hEPO mRNA encapsulated in lipid nanoparticle formulation made by Process A or by Process B.

[0057] **Figure 16** depicts an exemplary dosing and testing scheme in *Spf*^{ash} mice that involved an ammonia challenge.

[0058] **Figure 17** depicts exemplary plasma ammonia levels in *Spf*^{ash} mice after treatment with different dose levels of hOTC mRNA-loaded lipid nanoparticles, each prepared via Process B, following an ammonia challenge with NH_4Cl.

[0059] **Figure 18** shows hOTC protein expression in *Spf*^{ash} mouse livers 24 hours after a single intravenous dose (i.e., 0.5 mg/kg, 0.16 mg/kg, 0.05 mg/kg, or 0.016 mg/kg) of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or by Process B.

[0060] **Figure 19** shows a comparison of hOTC mRNA copy number in liver tissue of OTC *spf*^{ash} mice 24 hours after a single intravenous a dose (i.e., 0.5 mg/kg, 0.16 mg/kg, 0.05 mg/kg, or 0.016 mg/kg) of hOTC mRNA encapsulated in lipid nanoparticle formulation made by Process A or by Process B.

[0061] **Figure 20** shows a comparison of hOTC mRNA copy number in RNA tested of OTC *spf*^{ash} mice 24 hours after a single intravenous dose (i.e., 0.5 mg/kg, 0.16 mg/kg, 0.05 mg/kg, and 0.016 mg/kg) of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or by Process B.

[0062] **Figure 21** shows plasma ammonia results 40 minutes after being subjected to an ammonia challenge in each of wildtype mice (WT), untreated *spf*^{ash} mice (Untreated), and *spf*^{ash} mice at 24 hours (Day 2), 48 hours (Day 3), 72 hours (Day 4), 96 hours (Day 5), 8 days (Day 8), 11 days (Day 11), and 15 days (Day 15) following administration of 1.0 mg/kg hOTC mRNA lipid nanoparticles produced by Process B.

[0063] **Figure 22** shows hOTC protein activity as measured by citrulline production in each of wildtype mice (WT), untreated *spf*^{ash} mice (Untreated), and *spf*^{ash} mice at 24 hours (Day 2), 48 hours (Day 3), 72 hours (Day 4), 96 hours (Day 5), 8 days (Day 8), 11 days (Day 11), and 15 days (Day 15) following administration of 1.0 mg/kg hOTC mRNA lipid nanoparticles produced by Process B.

[0064] **Figure 23** shows hOTC protein activity as measured by maintained low levels of urinary orotic acid production in each of untreated *spf*^{ash} mice (Untreated), *spf*^{ash} mice at 24 hours (Day 2), 48 hours (Day 3), 72 hours (Day 4), 96 hours (Day 5), 8 days (Day 8), 11 days

(Day 11), and 15 days (Day 15) following administration of 1.0 mg/kg hOTC mRNA lipid nanoparticles produced by Process B, and untreated wildtype mice (Untreated C57BL/6).

[0065] **Figure 24** depicts exemplary activity of expressed hOTC protein (in terms of citrulline production) in livers of OTC spf^{ash} mice 24 hours after a single intravenous dose at different dose levels of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or by Process B.

[0066] **Figure 25** depicts the immunohistochemical detection of expressed hOTC protein in the mice livers by Western blot after a single intravenous dose of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or by Process B at various dosing levels.

[0067] **Figure 26** depicts exemplary activity of expressed hOTC protein (in terms of citrulline production) in livers of OTC *spf^{ash}* mice 24 hours after a single intravenous 0.5 mg/kg dose of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process B, as compared to those made by Process A.

[0068] **Figure 27(a)-(d)** shows the immunohistochemical detection of hOTC protein in mouse liver tissue 24 hours after dosing of hOTC mRNA lipid nanoparticles prepared by Process A or by Process B via immunohistochemical staining. **Figure 27(a)-(b)** depicts results from mRNA lipid nanoparticles produced by Process B. **Figure 27(c)-(d)** depicts results from mRNA lipid nanoparticles produced by Process A.

[0069] **Figure 28** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using HGT 5001 as the cationic lipid.

[0070] **Figure 29** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using ICE as the cationic lipid.

[0071] **Figure 30** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using cKK-E12 as the cationic lipid.

[0072] **Figure 31** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using C12-200 as the cationic lipid.

[0073] **Figure 32** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using HGT 4003 as the cationic lipid.

DEFINITIONS

[0074] In order for the present invention to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms are set forth throughout the specification.

[0075] *Alkyl*: As used herein, "alkyl" refers to a radical of a straight-chain or branched saturated hydrocarbon group having from 1 to 20 carbon atoms ("C_{1-20} alkyl"). In some embodiments, an alkyl group has 1 to 3 carbon atoms ("C_{1-3} alkyl"). Examples of C_{1-3} alkyl groups include methyl (C_1), ethyl (C_2), n-propyl (C_3), and isopropyl (C_3). In some embodiments, an alkyl group has 8 to 12 carbon atoms ("C_{8-12} alkyl"). Examples of C_{8-12} alkyl groups include, without limitation, *n*-octyl (C_8), *n*-nonyl (C_9), *n*-decyl (C_{10}), *n*-undecyl (C_{11}), *n*-dodecyl (C_{12}) and the like. The prefix "*n*-" (normal) refers to unbranched alkyl groups. For example, *n*-C_8 alkyl refers to -$(CH_2)_7CH_3$, *n*-C_{10} alkyl refers to -$(CH_2)_9CH_3$, etc.

[0076] *Amino acid*: As used herein, term "amino acid," in its broadest sense, refers to any compound and/or substance that can be incorporated into a polypeptide chain. In some embodiments, an amino acid has the general structure H_2N–C(H)(R)–COOH. In some embodiments, an amino acid is a naturally occurring amino acid. In some embodiments, an amino acid is a synthetic amino acid; in some embodiments, an amino acid is a d-amino acid; in some embodiments, an amino acid is an l-amino acid. "Standard amino acid" refers to any of the twenty standard l-amino acids commonly found in naturally occurring peptides. "Nonstandard amino acid" refers to any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or obtained from a natural source. As used

herein, "synthetic amino acid" encompasses chemically modified amino acids, including but not limited to salts, amino acid derivatives (such as amides), and/or substitutions. Amino acids, including carboxy- and/or amino-terminal amino acids in peptides, can be modified by methylation, amidation, acetylation, protecting groups, and/or substitution with other chemical groups that can change the peptide's circulating half-life without adversely affecting their activity. Amino acids may participate in a disulfide bond. Amino acids may comprise one or posttranslational modifications, such as association with one or more chemical entities (*e.g.*, methyl groups, acetate groups, acetyl groups, phosphate groups, formyl moieties, isoprenoid groups, sulfate groups, polyethylene glycol moieties, lipid moieties, carbohydrate moieties, biotin moieties, *etc.*). The term "amino acid" is used interchangeably with "amino acid residue," and may refer to a free amino acid and/or to an amino acid residue of a peptide. It will be apparent from the context in which the term is used whether it refers to a free amino acid or a residue of a peptide.

[0077] *Animal*: As used herein, the term "animal" refers to any member of the animal kingdom. In some embodiments, "animal" refers to humans, at any stage of development. In some embodiments, "animal" refers to non-human animals, at any stage of development. In certain embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, insects, and/or worms. In some embodiments, an animal may be a transgenic animal, genetically-engineered animal, and/or a clone.

[0078] *Approximately or about:* As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0079] *Delivery*: As used herein, the term "delivery" encompasses both local and systemic delivery. For example, delivery of mRNA encompasses situations in which an

mRNA is delivered to a target tissue and the encoded protein or peptide is expressed and retained within the target tissue (also referred to as "local distribution" or "local delivery"), and situations in which an mRNA is delivered to a target tissue and the encoded protein or peptide is expressed and secreted into patient's circulation system (e.g., serum) and systematically distributed and taken up by other tissues (also referred to as "systemic distribution" or "systemic delivery).

[0080] *Efficacy*: As used herein, the term "efficacy," or grammatical equivalents, refers to an improvement of a biologically relevant endpoint, as related to delivery of mRNA that encodes a relevant protein or peptide. In some embodiments, the biological endpoint is protecting against an ammonium chloride challenge at certain timepoints after administration.

[0081] *Encapsulation:* As used herein, the term "encapsulation," or grammatical equivalent, refers to the process of confining an individual mRNA molecule within a nanoparticle.

[0082] *Expression*: As used herein, "expression" of a mRNA refers to translation of an mRNA into a peptide (e.g., an antigen), polypeptide, or protein (e.g., an enzyme) and also can include, as indicated by context, the post-translational modification of the peptide, polypeptide or fully assembled protein (e.g., enzyme). In this application, the terms "expression" and "production," and grammatical equivalent, are used inter-changeably.

[0083] *Improve, increase, or reduce*: As used herein, the terms "improve," "increase" or "reduce," or grammatical equivalents, indicate values that are relative to a baseline measurement, such as a measurement in the same individual prior to initiation of the treatment described herein, or a measurement in a control sample or subject (or multiple control samples or subjects) in the absence of the treatment described herein. A "control sample" is a sample subjected to the same conditions as a test sample, except for the test article. A "control subject" is a subject afflicted with the same form of disease as the subject being treated, who is about the same age as the subject being treated.

[0084] *Impurities*: As used herein, the term "impurities" refers to substances inside a confined amount of liquid, gas, or solid, which differ from the chemical composition of the target material or compound. Impurities are also referred to as contaminants.

[0085] *In Vitro*: As used herein, the term "*in vitro*" refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, *etc.*, rather than within a multi-cellular organism.

[0086] *In Vivo*: As used herein, the term "*in vivo*" refers to events that occur within a multi-cellular organism, such as a human and a non-human animal. In the context of cell-based systems, the term may be used to refer to events that occur within a living cell (as opposed to, for example, *in vitro* systems).

[0087] *Isolated*: As used herein, the term "isolated" refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is "pure" if it is substantially free of other components. As used herein, calculation of percent purity of isolated substances and/or entities should not include excipients (*e.g.*, buffer, solvent, water, *etc.*).

[0088] *Local distribution or delivery*: As used herein, the terms "local distribution," "local delivery," or grammatical equivalent, refer to tissue specific delivery or distribution. Typically, local distribution or delivery requires a peptide or protein (e.g., enzyme) encoded by mRNAs be translated and expressed intracellularly or with limited secretion that avoids entering the patient's circulation system.

[0089] *messenger RNA (mRNA):* As used herein, the term "messenger RNA (mRNA)" refers to a polynucleotide that encodes at least one peptide, polypeptide or protein. mRNA as used herein encompasses both modified and unmodified RNA. mRNA may contain one or more coding and non-coding regions. mRNA can be purified from natural

sources, produced using recombinant expression systems and optionally purified, chemically synthesized, *etc.* Where appropriate, *e.g.*, in the case of chemically synthesized molecules, mRNA can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, backbone modifications, *etc.* An mRNA sequence is presented in the 5' to 3' direction unless otherwise indicated. In some embodiments, an mRNA is or comprises natural nucleosides (*e.g.*, adenosine, guanosine, cytidine, uridine); nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, 2-thiocytidine, pseudouridine, and 5-methylcytidine); chemically modified bases; biologically modified bases (*e.g.*, methylated bases); intercalated bases; modified sugars (*e.g.*, 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (*e.g.*, phosphorothioates and 5'-*N*-phosphoramidite linkages).

[0090] *Nucleic acid:* As used herein, the term "nucleic acid," in its broadest sense, refers to any compound and/or substance that is or can be incorporated into a polynucleotide chain. In some embodiments, a nucleic acid is a compound and/or substance that is or can be incorporated into a polynucleotide chain via a phosphodiester linkage. In some embodiments, "nucleic acid" refers to individual nucleic acid residues (e.g., nucleotides and/or nucleosides). In some embodiments, "nucleic acid" refers to a polynucleotide chain comprising individual nucleic acid residues. In some embodiments, "nucleic acid" encompasses RNA as well as single and/or double-stranded DNA and/or cDNA. Furthermore, the terms "nucleic acid," "DNA," "RNA," and/or similar terms include nucleic acid analogs, i.e., analogs having other than a phosphodiester backbone.

[0091] *Patient:* As used herein, the term "patient" or "subject" refers to any organism to which a provided composition may be administered, *e.g.*, for experimental, diagnostic, prophylactic, cosmetic, and/or therapeutic purposes. Typical patients include animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates, and/or humans). In some embodiments, a patient is a human. A human includes pre- and post-natal forms.

[0092] *Pharmaceutically acceptable*: The term "pharmaceutically acceptable" as used herein, refers to substances that, within the scope of sound medical judgment, are suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0093] *Pharmaceutically acceptable salt*: Pharmaceutically acceptable salts are well known in the art. For example, S. M. Berge et al., describes pharmaceutically acceptable salts in detail in *J. Pharmaceutical Sciences* (1977) 66:1-19. Pharmaceutically acceptable salts of the compounds of this invention include those derived from suitable inorganic and organic acids and bases. Examples of pharmaceutically acceptable, nontoxic acid addition salts are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or rnalonic acid or by using other methods used in the art such as ion exchange. Other pharmaceutically acceptable salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hemisulfate, heptanoate, hexanoate, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2- naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like. Salts derived from appropriate bases include alkali metal, alkaline earth metal, ammonium and $N^+(C_{1-4}$ alkyl$)_4$ salts. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium. quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, sulfonate and aryl sulfonate. Further pharmaceutically acceptable salts include salts formed from the quarternization of an amine

using an appropriate electrophile, e.g., an alkyl halide, to form a quarternized alkylated amino salt.

[0094] *Potency*: As used herein, the term "potency," or grammatical equivalents, refers to expression of protein(s) or peptide(s) that the mRNA encodes and/or the resulting biological effect.

[0095] *Salt*: As used herein the term "salt" refers to an ionic compound that does or may result from a neutralization reaction between an acid and a base.

[0096] *Systemic distribution or delivery*: As used herein, the terms "systemic distribution," "systemic delivery," or grammatical equivalent, refer to a delivery or distribution mechanism or approach that affect the entire body or an entire organism. Typically, systemic distribution or delivery is accomplished via body's circulation system, *e.g.*, blood stream. Compared to the definition of "local distribution or delivery."

[0097] *Subject*: As used herein, the term "subject" refers to a human or any non-human animal *(e.g.*, mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate). A human includes pre- and post-natal forms. In many embodiments, a subject is a human being. A subject can be a patient, which refers to a human presenting to a medical provider for diagnosis or treatment of a disease. The term "subject" is used herein interchangeably with "individual" or "patient." A subject can be afflicted with or is susceptible to a disease or disorder but may or may not display symptoms of the disease or disorder.

[0098] *Substantially*: As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0099] *Target tissues*: As used herein, the term "target tissues" refers to any tissue that is affected by a disease to be treated. In some embodiments, target tissues include those tissues that display disease-associated pathology, symptom, or feature.

[0100] *Treating*: As used herein, the term "treat," "treatment," or "treating" refers to any method used to partially or completely alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of and/or reduce incidence of one or more symptoms or features of a particular disease, disorder, and/or condition. Treatment may be administered to a subject who does not exhibit signs of a disease and/or exhibits only early signs of the disease for the purpose of decreasing the risk of developing pathology associated with the disease.

[0101] *Yield*: As used herein, the term "yield" refers to the percentage of mRNA recovered after encapsulation as compared to the total mRNA as starting material. In some embodiments, the term "recovery" is used interchangeably with the term "yield".

DETAILED DESCRIPTION

[0102] The present invention provides an improved process for lipid nanoparticle formulation and mRNA encapsulation. In some embodiments, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising the steps of forming lipids into pre-formed lipid nanoparticles (i.e., formed in the absence of mRNA) and then combining the pre-formed lipid nanoparticles with mRNA. In some embodiments, the novel formulation process results in an mRNA formulation with higher potency (peptide or protein expression) and higher efficacy (improvement of a biologically relevant endpoint) both *in vitro* and *in vivo* with potentially better tolerability as compared to the same mRNA formulation prepared without the step of preforming the lipid nanoparticles (e.g., combining the lipids directly with the mRNA). The higher potency and/or efficacy of such a formulation can provide for lower and/or less frequent dosing of the drug product. In some embodiments, the invention features an improved lipid formulation comprising a cationic lipid, a helper lipid and a PEG or PEG-modified lipid.

[0103] In some embodiments, the resultant encapsulation efficiencies for the present lipid nanoparticle formulation and preparation method are around 90%. For the delivery of nucleic acids, achieving high encapsulation efficiencies is critical to attain protection of the drug substance and reduce loss of activity *in vivo*. In addition, a surprising result for the lipid

nanoparticle formulation prepared by the novel method in the current invention is the significantly higher transfection efficiency observed *in vitro*.

[0104] Various aspects of the invention are described in detail in the following sections. The use of sections is not meant to limit the invention. Each section can apply to any aspect of the invention.

Messenger RNA (mRNA)

[0105] The present invention may be used to encapsulate any mRNA. mRNA is typically thought of as the type of RNA that carries information from DNA to the ribosome. Typically, in eukaryotic organisms, mRNA processing comprises the addition of a "cap" on the 5' end, and a "tail" on the 3' end. A typical cap is a 7-methylguanosine cap, which is a guanosine that is linked through a 5'-5'-triphosphate bond to the first transcribed nucleotide. The presence of the cap is important in providing resistance to nucleases found in most eukaryotic cells. The additional of a tail is typically a polyadenylation event whereby a polyadenylyl moiety is added to the 3' end of the mRNA molecule. The presence of this "tail" serves to protect the mRNA from exonuclease degradation. Messenger RNA is translated by the ribosomes into a series of amino acids that make up a protein.

[0106] mRNAs may be synthesized according to any of a variety of known methods. For example, mRNAs according to the present invention may be synthesized via *in vitro* transcription (IVT). Briefly, IVT is typically performed with a linear or circular DNA template containing a promoter, a pool of ribonucleotide triphosphates, a buffer system that may include DTT and magnesium ions, and an appropriate RNA polymerase (e.g., T3, T7 or SP6 RNA polymerase), DNAse I, pyrophosphatase, and/or RNAse inhibitor. The exact conditions will vary according to the specific application.

[0107] In some embodiments, *in vitro* synthesized mRNA may be purified before formulation and encapsulation to remove undesirable impurities including various enzymes and other reagents used during mRNA synthesis.

[0108] The present invention may be used to formulate and encapsulate mRNAs of a variety of lengths. In some embodiments, the present invention may be used to formulate and encapsulate *in vitro* synthesized mRNA of or greater than about 1 kb, 1.5 kb, 2 kb, 2.5 kb, 3 kb, 3.5 kb, 4 kb, 4.5 kb, 5 kb 6 kb, 7 kb, 8 kb, 9 kb, 10 kb, 11 kb, 12 kb, 13 kb, 14 kb, 15 kb, or 20 kb in length. In some embodiments, the present invention may be used to formulate and encapsulate *in vitro* synthesized mRNA ranging from about 1-20 kb, about 1-15 kb, about 1-10 kb, about 5-20 kb, about 5-15 kb, about 5-12 kb, about 5-10 kb, about 8-20 kb, or about 8-15 kb in length.

[0109] The present invention may be used to formulate and encapsulate mRNA that is unmodified or mRNA containing one or more modifications that typically enhance stability. In some embodiments, modifications are selected from modified nucleotides, modified sugar phosphate backbones, and 5' and/or 3' untranslated region.

[0110] In some embodiments, modifications of mRNA may include modifications of the nucleotides of the RNA. A modified mRNA according to the invention can include, for example, backbone modifications, sugar modifications or base modifications. In some embodiments, mRNAs may be synthesized from naturally occurring nucleotides and/or nucleotide analogues (modified nucleotides) including, but not limited to, purines (adenine (A), guanine (G)) or pyrimidines (thymine (T), cytosine (C), uracil (U)), and as modified nucleotides analogues or derivatives of purines and pyrimidines, such as e.g. 1-methyl-adenine, 2-methyl-adenine, 2-methylthio-N-6-isopentenyl-adenine, N6-methyl-adenine, N6-isopentenyl-adenine, 2-thio-cytosine, 3-methyl-cytosine, 4-acetyl-cytosine, 5-methyl-cytosine, 2,6-diaminopurine, 1-methyl-guanine, 2-methyl-guanine, 2,2-dimethyl-guanine, 7-methyl-guanine, inosine, 1-methyl-inosine, pseudouracil (5-uracil), dihydro-uracil, 2-thio-uracil, 4-thio-uracil, 5-carboxymethylaminomethyl-2-thio-uracil, 5-(carboxyhydroxymethyl)-uracil, 5-fluoro-uracil, 5-bromo-uracil, 5-carboxymethylaminomethyl-uracil, 5-methyl-2-thio-uracil, 5-methyl-uracil, N-uracil-5-oxyacetic acid methyl ester, 5-methylaminomethyl-uracil, 5-methoxyaminomethyl-2-thio-uracil, 5'-methoxycarbonylmethyl-uracil, 5-methoxy-uracil, uracil-5-oxyacetic acid methyl ester, uracil-5-oxyacetic acid (v), 1-methyl-pseudouracil, queosine, .beta.-D-mannosyl-queosine, wybutoxosine, and phosphoramidates, phosphorothioates, peptide nucleotides, methylphosphonates, 7-deazaguanosine, 5-

methylcytosine, pseudouridine, 5-methylcytidine and inosine. The preparation of such analogues is known to a person skilled in the art e.g. from the U.S. Pat. No. 4,373,071, U.S. Pat. No. 4,401,796, U.S. Pat. No. 4,415,732, U.S. Pat. No. 4,458,066, U.S. Pat. No. 4,500,707, U.S. Pat. No. 4,668,777, U.S. Pat. No. 4,973,679, U.S. Pat. No. 5,047,524, U.S. Pat. No. 5,132,418, U.S. Pat. No. 5,153,319, U.S. Pat. Nos. 5,262,530 and 5,700,642, the disclosure of which is included here in its full scope by reference.

[0111] Typically, mRNA synthesis includes the addition of a "cap" on the 5' end, and a "tail" on the 3' end. The presence of the cap is important in providing resistance to nucleases found in most eukaryotic cells. The presence of a "tail" serves to protect the mRNA from exonuclease degradation.

[0112] Thus, in some embodiments, mRNAs include a 5' cap structure. A 5' cap is typically added as follows: first, an RNA terminal phosphatase removes one of the terminal phosphate groups from the 5' nucleotide, leaving two terminal phosphates; guanosine triphosphate (GTP) is then added to the terminal phosphates via a guanylyl transferase, producing a 5'5'5 triphosphate linkage; and the 7-nitrogen of guanine is then methylated by a methyltransferase. 2'-O-methylation may also occur at the first base and/or second base following the 7-methyl guanosine triphosphate residues. Examples of cap structures include, but are not limited to, m7GpppNp-RNA , m7GpppNmp-RNA and m7GpppNmpNmp-RNA (where m indicates 2'-Omethyl residues).

[0113] In some embodiments, mRNAs include a 5' and/or 3' untranslated region. In some embodiments, a 5' untranslated region includes one or more elements that affect an mRNA's stability or translation, for example, an iron responsive element. In some embodiments, a 5' untranslated region may be between about 50 and 500 nucleotides in length.

[0114] In some embodiments, a 3' untranslated region includes one or more of a polyadenylation signal, a binding site for proteins that affect an mRNA's stability of location in a cell, or one or more binding sites for miRNAs. In some embodiments, a 3' untranslated region may be between 50 and 500 nucleotides in length or longer.

[0115] While mRNA provided from *in vitro* transcription reactions may be desirable in some embodiments, other sources of mRNA are contemplated as within the scope of the invention including mRNA produced from bacteria, fungi, plants, and/or animals.

[0116] The present invention may be used to formulate and encapsulate mRNAs encoding a variety of proteins. Non-limiting examples of mRNAs suitable for the present invention include mRNAs encoding spinal motor neuron 1 (SMN), alpha-galactosidase (GLA), argininosuccinate synthetase (ASS1), ornithine transcarbamylase (OTC), Factor IX (FIX), phenylalanine hydroxylase (PAH), erythropoietin (EPO), cystic fibrosis transmembrane conductance receptor (CFTR) and firefly luciferase (FFL). Exemplary mRNA sequences as disclosed herein are listed below:

Codon-Optimized Human OTC Coding Sequence

AUGCUGUUCAACCUUCGGAUCUUGCUGAACAACGCUGCGUUCCGGAAUGGUCA
CAACUUCAUGGUCCGGAACUUCAGAUGCGGCCAGCCGCUCCAGAACAAGGUGC
AGCUCAAGGGGAGGGACCUCCUCACCCUGAAAAACUUCACCGGAGAAGAGAUC
AAGUACAUGCUGUGGCUGUCAGCCGACCUCAAAUUCCGGAUCAAGCAGAAGGG
CGAAUACCUUCCUUUGCUGCAGGGAAAGUCCCUGGGGAUGAUCUUCGAGAAGC
GCAGCACUCGCACUAGACUGUCAACUGAAACCGGCUUCGCGCUGCUGGGAGGA
CACCCCUGCUUCCUGACCACCCAAGAUAUCCAUCUGGGUGUGAACGAAUCCCU
CACCGACACAGCGCGGGUGCUGUCGUCCAUGGCAGACGCGGUCCUCGCCCGCG
UGUACAAGCAGUCUGAUCUGGACACUCUGGCCAAGGAAGCCUCCAUUCCUAUC
AUUAAUGGAUUGUCCGACCUCUACCAUCCCAUCCAGAUUCUGGCCGAUUAUCU
GACUCUGCAAGAACAUUACAGCUCCCUGAAGGGGCUUACCCUUUCGUGGAUCG
GCGACGGCAACAACAUUCUGCACAGCAUUAUGAUGAGCGCUGCCAAGUUUGGA
AUGCACCUCCAAGCAGCGACCCCGAAGGGAUACGAGCCAGACGCCUCCGUGAC
GAAGCUGGCUGAGCAGUACGCCAAGGAGAACGGCACUAAGCUGCUGCUCACCA
ACGACCCUCUCGAAGCCGCCCACGGUGGCAACGUGCUGAUCACCGAUACCUGG
AUCUCCAUGGGACAGGAGGAGGAAAAGAAGAAGCGCCUGCAAGCAUUUCAGG
GGUACCAGGUGACUAUGAAAACCGCCAAGGUCGCCGCCUCGGACUGGACCUUC
UUGCACUGUCUGCCCAGAAAGCCCGAAGAGGUGGACGACGAGGUGUUCUACAG
CCCGCGGUCGCUGGUCUUUCCGGAGGCCGAAAACAGGAAGUGGACUAUCAUGG

CCGUGAUGGUGUCCCUGCUGACCGAUUACUCCCCGCAGCUGCAGAAACCAAAG
UUCUGA (**SEQ ID NO: 1**)

Codon-Optimized Human ASS1 Coding Sequence

AUGAGCAGCAAGGGCAGCGUGGUGCUGGCCUACAGCGGCGGCCUGGACACCAG
CUGCAUCCUGGUGUGGCUGAAGGAGCAGGGCUACGACGUGAUCGCCUACCUGG
CCAACAUCGGCCAGAAGGAGGACUUCGAGGAGGCCCGCAAGAAGGCCCUGAAG
CUGGGCGCCAAGAAGGUGUUCAUCGAGGACGUGAGCCGCGAGUUCGUGGAGG
AGUUCAUCUGGCCCGCCAUCCAGAGCAGCGCCCUGUACGAGGACCGCUACCUG
CUGGGCACCAGCCUGGCCCGCCCCUGCAUCGCCCGCAAGCAGGUGGAGAUCGC
CCAGCGCGAGGGCGCCAAGUACGUGAGCCACGGCGCCACCGGCAAGGGCAACG
ACCAGGUGCGCUUCGAGCUGAGCUGCUACAGCCUGGCCCCCCAGAUCAAGGUG
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CCUGAUGGAGUACGCCAAGCAGCACGGCAUCCCCAUCCCCGUGACCCCCAAGA
ACCCCUGGAGCAUGGACGAGAACCUGAUGCACAUCAGCUACGAGGCCGGCAUC
CUGGAGAACCCCAAGAACCAGGCCCCCCCCGGCCUGUACACCAAGACCCAGGA
CCCCGCCAAGGCCCCCAACACCCCCGACAUCCUGGAGAUCGAGUUCAAGAAGG
GCGUGCCCGUGAAGGUGACCAACGUGAAGGACGGCACCACCCACCAGACCAGC
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ACGAGGAGCUGGUGAGCAUGAACGUGCAGGGCGACUACGAGCCCACCGACGCC
ACCGGCUUCAUCAACAUCAACAGCCUGCGCCUGAAGGAGUACCACCGCCUGCA
GAGCAAGGUGACCGCCAAGUGA (**SEQ ID NO: 2**)

Codon-Optimized Human CFTR Coding Sequence

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GAGAGAGAAUGGGACAGAGAACUCGCCUCAAAGAAGAACCCGAAGCUGAUUA
AUGCGCUUAGGCGGUGCUUUUUCUGGCGGUUCAUGUUCUACGGCAUCUUCCUC
UACCUGGGAGAGGUCACCAAGGCCGUGCAGCCCCUGUUGCUGGGACGGAUUAU
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GCAUCGGUCUGUGCCUGCUUUUCAUCGUCCGGACCCUCUUGUUGCAUCCUGCU
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GAUCUACAAGAAAACUCUGAAGCUCUCGAGCCGCGUGCUUGACAAGAUUUCCA
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GGGCCUGAUCUGGGAGCUGCUGCAAGCCUCGGCAUUCUGUGGGCUUGGAUUCC
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ACGGUGACGACUCCCUCUUCUUUUCAAACUUCAGCCUGCUCGGGACGCCCGUG
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UGAACCUAGCGAGGGAAAGAUCAAGCACUCCGGCCGCAUCAGCUUCUGUAGCC
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CUGGUCCGACGCUCCAAGCCCGGCGGCGGCAGUCCGUGCUGAACCUGAUGACC
CACAGCGUGAACCAGGGCCAAAACAUUCACCGCAAGACUACCGCAUCCACCCG
GAAAGUGUCCCUGGCACCUCAAGCGAAUCUUACCGAGCUCGACAUCUACUCCC
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GGAUCUGAAAGAGUGCUUCUUCGACGAUAUGGAGUCGAUACCCGCCGUGACG
ACUUGGAACACUUAUCUGCGGUACAUCACUGUGCACAAGUCAUUGAUCUUCGU
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UCCUGCCGCUUACCAUCUUUGACUUCAUCCAGCUGCUGCUGAUCGUGAUUGGA
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UUCAAAAGCUCCUCAACGAGCGGUCGCUGUUCAGACAAGCUAUUUCACCGUCC
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GCAGAUCGCAGCCUUGAAGGAAGAGACUGAGGAAGAGGUGCAGGACACCCGG
CUU**UAA (SEQ ID NO: 3)**

Comparison Codon-Optimized Human CFTR mRNA Coding Sequence

AUGCAGCGGUCCCCGCUCGAAAAGGCCAGUGUCGUGUCCAAACUCUUCUUCUC
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GACAUCUACCAGAUCCCCUCGGUAGAUUCGGCGGAUAACCUCUCGGAGAAGCU
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CCAUCGGUCAGUUGGUGUCCCUGCUUAGUAAUAACCUCAACAAAUUCGAUGAG

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UCGCGACCGUUCCGGUGAUCGUGGCGUUUAUCAUGCUGCGGGCCUAUUUCUUG

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Codon-Optimized Human PAH Coding Sequence

AUGAGCACCGCCGUGCUGGAGAACCCCGGCCUGGGCCGCAAGCUGAGCGACUU
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GACAACACCCAGCAGCUGAAGAUCCUGGCCGACAGCAUCAACAGCGAGAUCGG
CAUCCUGUGCAGCGCCCUGCAGAAGAUCAAGUAA (**SEQ ID NO: 5**)

[0117] In some embodiments, an mRNA suitable for the present invention has a nucleotide sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3 or SEQ ID NO: 4. In some embodiments, an mRNA suitable for the present invention comprises a nucleotide sequence identical to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3 or SEQ ID NO: 4.

mRNA Solution

[0118] mRNA may be provided in a solution to be mixed with a lipid solution such that the mRNA may be encapsulated in lipid nanoparticles. A suitable mRNA solution may be any aqueous solution containing mRNA to be encapsulated at various concentrations. For example, a suitable mRNA solution may contain an mRNA at a concentration of or greater than about 0.01 mg/ml, 0.05 mg/ml, 0.06 mg/ml, 0.07 mg/ml, 0.08 mg/ml, 0.09 mg/ml, 0.1 mg/ml, 0.15 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, or 1.0 mg/ml. In some embodiments, a suitable mRNA solution may

contain an mRNA at a concentration ranging from about 0.01-1.0 mg/ml, 0.01-0.9 mg/ml, 0.01-0.8 mg/ml, 0.01-0.7 mg/ml, 0.01-0.6 mg/ml, 0.01-0.5 mg/ml, 0.01-0.4 mg/ml, 0.01-0.3 mg/ml, 0.01-0.2 mg/ml, 0.01-0.1 mg/ml, 0.05-1.0 mg/ml, 0.05-0.9 mg/ml, 0.05-0.8 mg/ml, 0.05-0.7 mg/ml, 0.05-0.6 mg/ml, 0.05-0.5 mg/ml, 0.05-0.4 mg/ml, 0.05-0.3 mg/ml, 0.05-0.2 mg/ml, 0.05-0.1 mg/ml, 0.1-1.0 mg/ml, 0.2-0.9 mg/ml, 0.3-0.8 mg/ml, 0.4-0.7 mg/ml, or 0.5-0.6 mg/ml. In some embodiments, a suitable mRNA solution may contain an mRNA at a concentration up to about 5.0 mg/ml, 4.0 mg/ml, 3.0 mg/ml, 2.0 mg/ml, 1.0 mg/ml, .09 mg/ml, 0.08 mg/ml, 0.07 mg/ml, 0.06 mg/ml, or 0.05 mg/ml.

[0119] Typically, a suitable mRNA solution may also contain a buffering agent and/or salt. Generally, buffering agents can include HEPES, ammonium sulfate, sodium bicarbonate, sodium citrate, sodium acetate, potassium phosphate and sodium phosphate. In some embodiments, suitable concentration of the buffering agent may range from about 0.1 mM to 100 mM, 0.5 mM to 90 mM, 1.0 mM to 80 mM, 2 mM to 70 mM, 3 mM to 60 mM, 4 mM to 50 mM, 5 mM to 40 mM, 6 mM to 30 mM, 7 mM to 20 mM, 8 mM to 15 mM, or 9 to 12 mM. In some embodiments, suitable concentration of the buffering agent is or greater than about 0.1 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, 6 mM, 8 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, or 50 mM.

[0120] Exemplary salts can include sodium chloride, magnesium chloride, and potassium chloride. In some embodiments, suitable concentration of salts in an mRNA solution may range from about 1 mM to 500 mM, 5 mM to 400 mM, 10 mM to 350 mM, 15 mM to 300 mM, 20 mM to 250 mM, 30 mM to 200 mM, 40 mM to 190 mM, 50 mM to 180 mM, 50 mM to 170 mM, 50 mM to 160 mM, 50 mM to 150 mM, or 50 mM to 100 mM. Salt concentration in a suitable mRNA solution is or greater than about 1 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, or 100 mM.

[0121] In some embodiments, a suitable mRNA solution may have a pH ranging from about 3.5-6.5, 3.5-6.0, 3.5-5.5., 3.5-5.0, 3.5-4.5, 4.0-5.5, 4.0-5.0, 4.0-4.9, 4.0-4.8, 4.0-4.7, 4.0-4.6, or 4.0-4.5. In some embodiments, a suitable mRNA solution may have a pH of or no greater than about 3.5, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 6.1, 6.3, and 6.5.

[0122] Various methods may be used to prepare an mRNA solution suitable for the present invention. In some embodiments, mRNA may be directly dissolved in a buffer solution described herein. In some embodiments, an mRNA solution may be generated by mixing an mRNA stock solution with a buffer solution prior to mixing with a lipid solution for encapsulation. In some embodiments, an mRNA solution may be generated by mixing an mRNA stock solution with a buffer solution immediately before mixing with a lipid solution for encapsulation. In some embodiments, a suitable mRNA stock solution may contain mRNA in water at a concentration at or greater than about 0.2 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml, 1.2 mg/ml, 1.4 mg/ml, 1.5 mg/ml, or 1.6 mg/ml, 2.0 mg/ml, 2.5 mg/ml, 3.0 mg/ml, 3.5 mg/ml, 4.0 mg/ml, 4.5 mg/ml, or 5.0 mg/ml.

[0123] In some embodiments, an mRNA stock solution is mixed with a buffer solution using a pump. Exemplary pumps include but are not limited to gear pumps, peristaltic pumps and centrifugal pumps.

[0124] Typically, the buffer solution is mixed at a rate greater than that of the mRNA stock solution. For example, the buffer solution may be mixed at a rate at least 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x, 15x, or 20x greater than the rate of the mRNA stock solution. In some embodiments, a buffer solution is mixed at a flow rate ranging between about 100-6000 ml/minute (e.g., about 100-300 ml/minute, 300-600 ml/minute, 600-1200 ml/minute, 1200-2400 ml/minute, 2400-3600 ml/minute, 3600-4800 ml/minute, 4800-6000 ml/minute, or 60-420 ml/minute). In some embodiments, a buffer solution is mixed at a flow rate of or greater than about 60 ml/minute, 100 ml/minute, 140 ml/minute, 180 ml/minute, 220 ml/minute, 260 ml/minute, 300 ml/minute, 340 ml/minute, 380 ml/minute, 420 ml/minute, 480 ml/minute, 540 ml/minute, 600 ml/minute, 1200 ml/minute, 2400 ml/minute, 3600 ml/minute, 4800 ml/minute, or 6000 ml/minute.

[0125] In some embodiments, an mRNA stock solution is mixed at a flow rate ranging between about 10-600 ml/minute (e.g., about 5-50 ml/minute, about 10-30 ml/minute, about 30-60 ml/minute, about 60-120 ml/minute, about 120-240 ml/minute, about 240-360 ml/minute, about 360-480 ml/minute, or about 480-600 ml/minute). In some embodiments, an mRNA stock solution is mixed at a flow rate of or greater than about 5 ml/minute, 10 ml/minute, 15 ml/minute, 20 ml/minute, 25 ml/minute, 30 ml/minute, 35

ml/minute, 40 ml/minute, 45 ml/minute, 50 ml/minute, 60 ml/minute, 80 ml/minute, 100 ml/minute, 200 ml/minute, 300 ml/minute, 400 ml/minute, 500 ml/minute, or 600 ml/minute.

Lipid Solution

[0126] According to the present invention, a lipid solution contains a mixture of lipids suitable to form lipid nanoparticles for encapsulation of mRNA. In some embodiments, a suitable lipid solution is ethanol based. For example, a suitable lipid solution may contain a mixture of desired lipids dissolved in pure ethanol (i.e., 100% ethanol). In another embodiment, a suitable lipid solution is isopropyl alcohol based. In another embodiment, a suitable lipid solution is dimethylsulfoxide-based. In another embodiment, a suitable lipid solution is a mixture of suitable solvents including, but not limited to, ethanol, isopropyl alcohol and dimethylsulfoxide.

[0127] A suitable lipid solution may contain a mixture of desired lipids at various concentrations. For example, a suitable lipid solution may contain a mixture of desired lipids at a total concentration of or greater than about 0.1 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, 3.0 mg/ml, 4.0 mg/ml, 5.0 mg/ml, 6.0 mg/ml, 7.0 mg/ml, 8.0 mg/ml, 9.0 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml, or 100 mg/ml. In some embodiments, a suitable lipid solution may contain a mixture of desired lipids at a total concentration ranging from about 0.1-100 mg/ml, 0.5-90 mg/ml, 1.0-80 mg/ml, 1.0-70 mg/ml, 1.0-60 mg/ml, 1.0-50 mg/ml, 1.0-40 mg/ml, 1.0-30 mg/ml, 1.0-20 mg/ml, 1.0-15 mg/ml, 1.0-10 mg/ml, 1.0-9 mg/ml, 1.0-8 mg/ml, 1.0-7 mg/ml, 1.0-6 mg/ml, or 1.0-5 mg/ml. In some embodiments, a suitable lipid solution may contain a mixture of desired lipids at a total concentration up to about 100 mg/ml, 90 mg/ml, 80 mg/ml, 70 mg/ml, 60 mg/ml, 50 mg/ml, 40 mg/ml, 30 mg/ml, 20 mg/ml, or 10 mg/ml.

[0128] Any desired lipids may be mixed at any ratios suitable for encapsulating mRNAs. In some embodiments, a suitable lipid solution contains a mixture of desired lipids including cationic lipids, helper lipids (e.*g.* non cationic lipids and/or cholesterol lipids) and/or PEGylated lipids. In some embodiments, a suitable lipid solution contains a mixture

of desired lipids including one or more cationic lipids, one or more helper lipids (e.g. non cationic lipids and/or cholesterol lipids) and one or more PEGylated lipids.

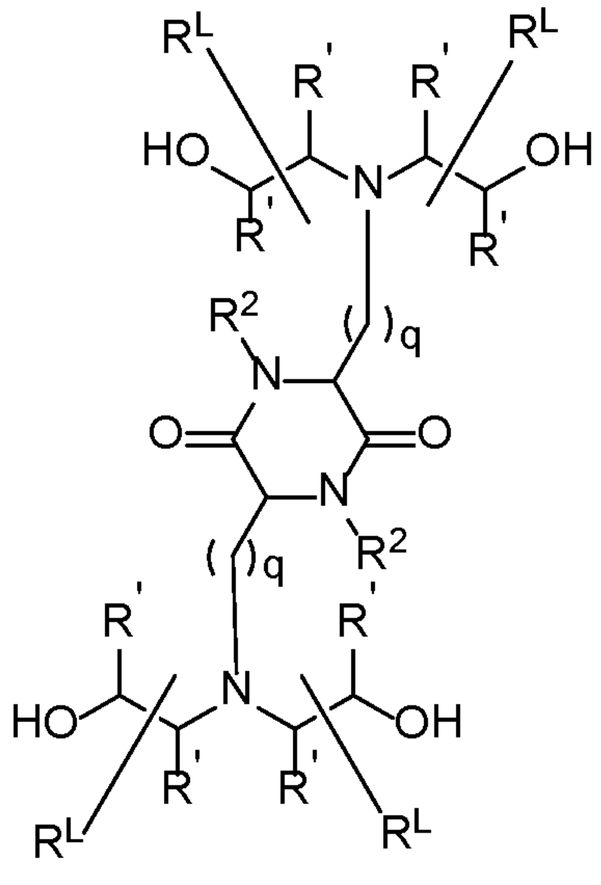
Cationic Lipids

[0129] As used herein, the phrase "cationic lipids" refers to any of a number of lipid species that have a net positive charge at a selected pH, such as physiological pH. Several cationic lipids have been described in the literature, many of which are commercially available. Particularly suitable cationic lipids for use in the compositions and methods of the invention include those described in international patent publications WO 2010/053572 (and particularly, C12-200 described at paragraph [00225]) and WO 2012/170930, both of which are incorporated herein by reference. In certain embodiments, cationic lipids suitable for the compositions and methods of the invention include an ionizable cationic lipid described in U.S. provisional patent application 61/617,468, filed March 29, 2012 (incorporated herein by reference), such as, e.g, (15Z, 18Z)-N,N-dimethyl-6-(9Z, 12Z)-octadeca-9, 12-dien-l -yl)tetracosa- 15,18-dien- 1 -amine (HGT5000), (15Z, 18Z)-N,N-dimethyl-6-((9Z, 12Z)-octadeca-9, 12-dien- 1 -yl)tetracosa-4,15,18-trien-l -amine (HGT5001), and (15Z,18Z)-N,N-dimethyl-6-((9Z, 12Z)-octadeca-9, 12-dien- 1 -yl)tetracosa-5, 15 , 18-trien- 1 -amine (HGT5002).

[0130] In some embodiments, cationic lipids suitable for the compositions and methods of the invention include cationic lipids such as 3,6-bis(4-(bis((9Z,12Z)-2-hydroxyoctadeca-9,12-dien-1-yl)amino)butyl)piperazine-2,5-dione (OF-02).

[0131] In some embodiments, cationic lipids suitable for the compositions and methods of the invention include a cationic lipid described in WO 2015/184256 A2 entitled "Biodegradable lipids for delivery of nucleic acids" which is incorporated by reference herein such as 3-(4-(bis(2-hydroxydodecyl)amino)butyl)-6-(4-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)butyl)-1,4-dioxane-2,5-dione (Target 23), 3-(5-(bis(2-hydroxydodecyl)amino)pentan-2-yl)-6-(5-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)pentan-2-yl)-1,4-dioxane-2,5-dione (Target 24).

[0132] In some embodiments, cationic lipids suitable for the compositions and methods of the invention include a cationic lipid described in WO 2013/063468 and in U.S. provisional application entitled "Lipid Formulations for Delivery of Messenger RNA", both of which are incorporated by reference herein. In some embodiments, a cationic lipid comprises a compound of formula **I-c1-a**:



I-c1-a,

or a pharmaceutically acceptable salt thereof, wherein:

each R^2 independently is hydrogen or C_{1-3} alkyl;

each q independently is 2 to 6;

each R' independently is hydrogen or C_{1-3} alkyl;

and each R^L independently is C_{8-12} alkyl.

[0133] In some embodiments, each R^2 independently is hydrogen, methyl or ethyl. In some embodiments, each R^2 independently is hydrogen or methyl. In some embodiments, each R^2 is hydrogen.

[0134] In some embodiments, each q independently is 3 to 6. In some embodiments, each q independently is 3 to 5. In some embodiments, each q is 4.

[0135] In some embodiments, each R' independently is hydrogen, methyl or ethyl. In some embodiments, each R' independently is hydrogen or methyl. In some embodiments, each R' independently is hydrogen.

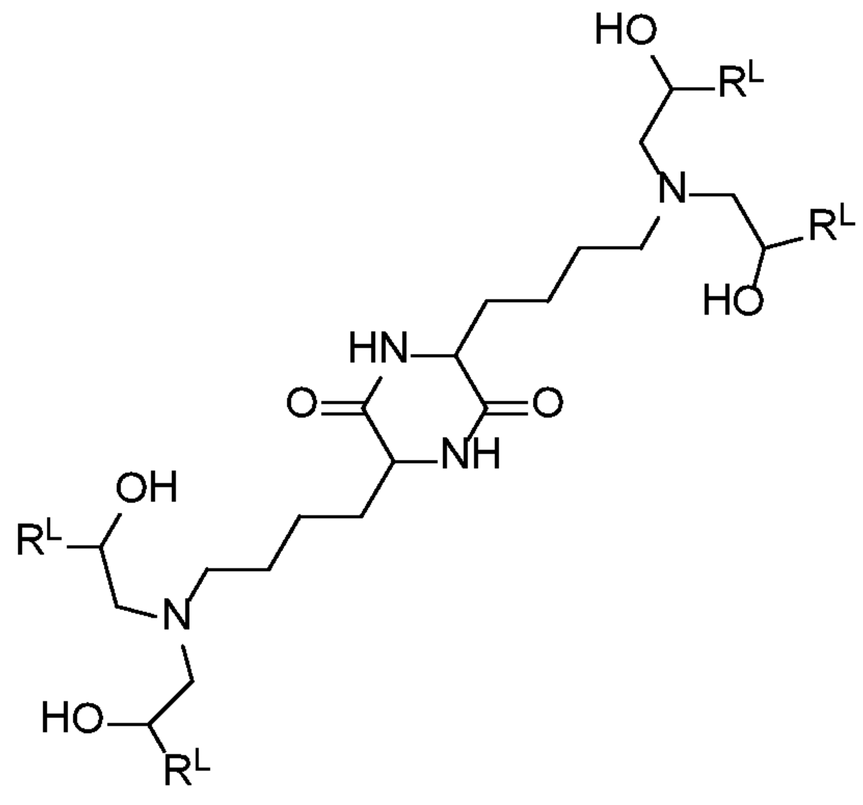
[0136] In some embodiments, each R^L independently is C_{8-12} alkyl. In some embodiments, each R^L independently is *n*-C_{8-12} alkyl. In some embodiments, each R^L independently is C_{9-11} alkyl. In some embodiments, each R^L independently is *n*-C_{9-11} alkyl. In some embodiments, each R^L independently is C_{10} alkyl. In some embodiments, each R^L independently is *n*-C_{10} alkyl.

[0137] In some embodiments, each R^2 independently is hydrogen or methyl; each q independently is 3 to 5; each R' independently is hydrogen or methyl; and each R^L independently is C_{8-12} alkyl.

[0138] In some embodiments, each R^2 is hydrogen; each q independently is 3 to 5; each R' is hydrogen; and each R^L independently is C_{8-12} alkyl.

[0139] In some embodiments, each R^2 is hydrogen; each q is 4; each R' is hydrogen; and each R^L independently is C_{8-12} alkyl.

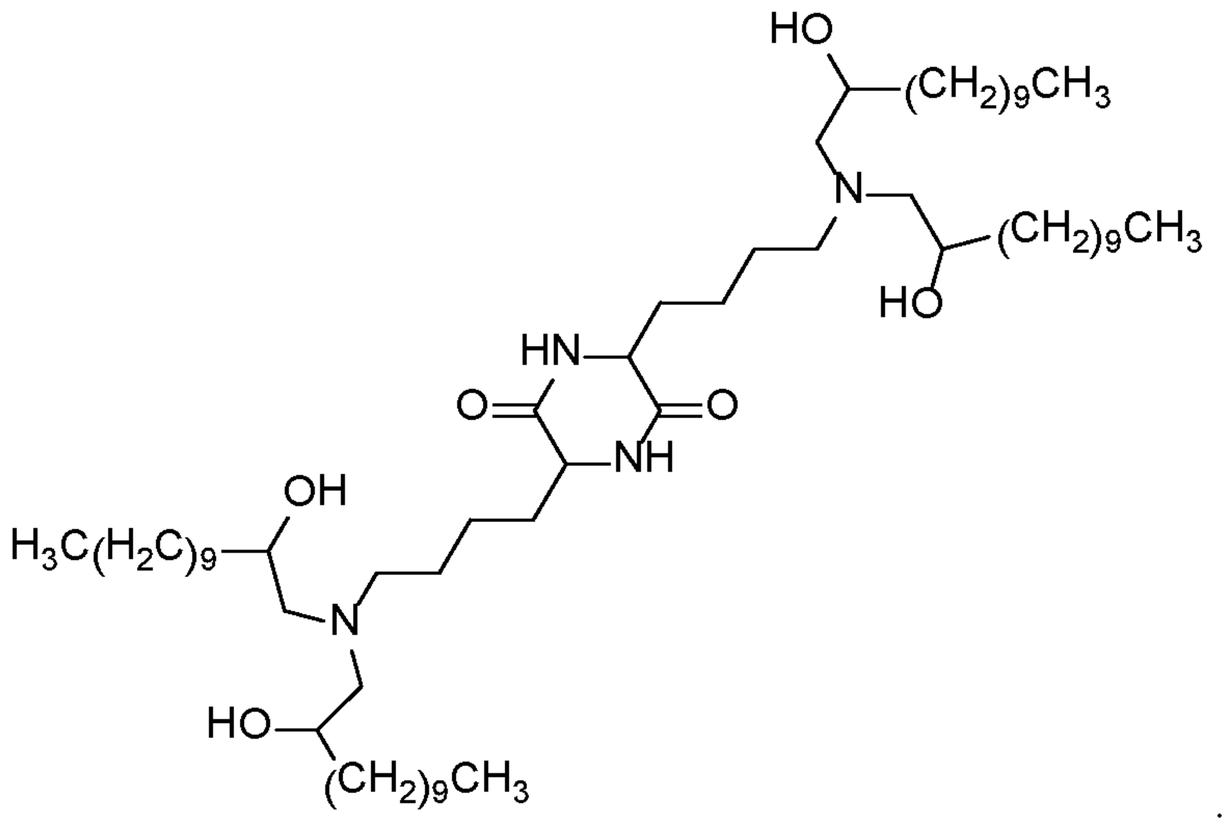
[0140] In some embodiments, a cationic lipid comprises a compound of formula **I-g**:



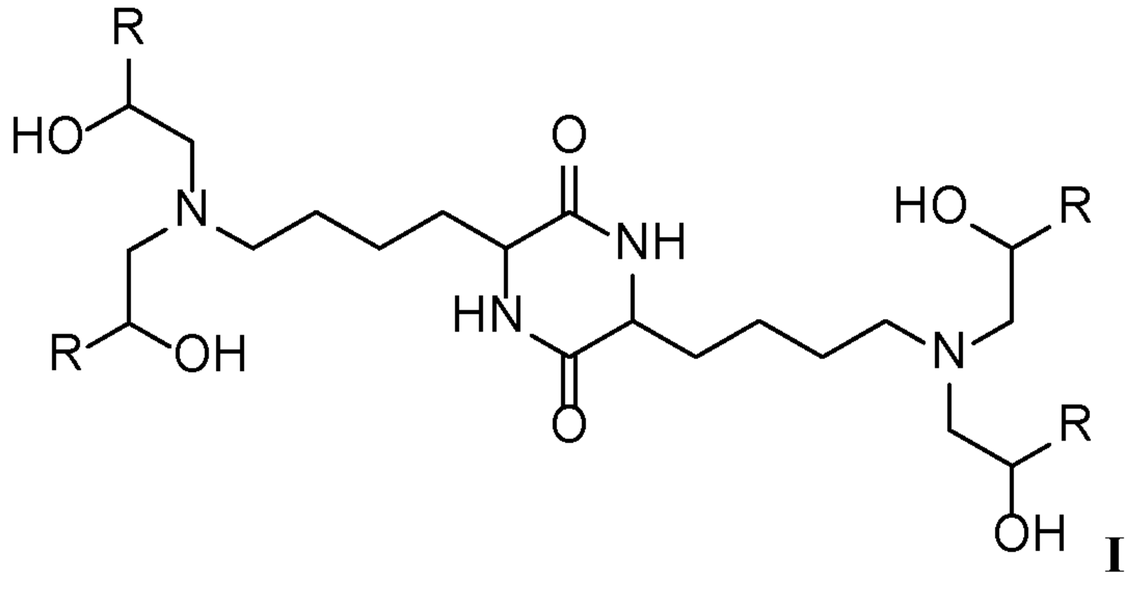
I-g,

or a pharmaceutically acceptable salt thereof, wherein each R^L independently is C_{8-12} alkyl. In some embodiments, each R^L independently is *n*-C_{8-12} alkyl. In some embodiments, each R^L independently is C_{9-11} alkyl. In some embodiments, each R^L independently is *n*-C_{9-11} alkyl. In some embodiments, each R^L independently is C_{10} alkyl. In some embodiments, each R^L is *n*-C_{10} alkyl.

[0141] In particular embodiments, a suitable cationic lipid is cKK-E12, or (3,6-bis(4-(bis(2-hydroxydodecyl)amino)butyl)piperazine-2,5-dione). Structure of cKK-E12 is shown below:



[0142] Additional exemplary cationic lipids include those of formula I:



I

and pharmaceutically acceptable salts thereof,

wherein,

R is ("OF-00"),

R is ("OF-01"),

R is ("OF-02"), or

R is ("OF-03")

(see, e.g., Fenton, Owen S., et al. "Bioinspired Alkenyl Amino Alcohol Ionizable Lipid Materials for Highly Potent In Vivo mRNA Delivery." *Advanced materials* (2016)).

[0143] In some embodiments, one or more cationic lipids suitable for the present invention may be N-[l-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride or "DOTMA". (Feigner et al. (Proc. Nat'l Acad. Sci. 84, 7413 (1987); U.S. Pat. No. 4,897,355). Other suitable cationic lipids include, for example, 5-carboxyspermylglycinedioctadecylamide or "DOGS," 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-l-propanaminium or "DOSPA" (Behr et al. Proc. Nat.'l Acad. Sci. 86, 6982 (1989); U.S. Pat. No. 5,171,678; U.S. Pat. No. 5,334,761), l,2-Dioleoyl-3-Dimethylammonium-Propane or "DODAP", l,2-Dioleoyl-3-Trimethylammonium-Propane or "DOTAP".

[0144] Additional exemplary cationic lipids also include l,2-distearyloxy-N,N-dimethyl-3-aminopropane or "DSDMA", 1,2-dioleyloxy-N,N-dimethyl-3-aminopropane or "DODMA", 1 ,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane or "DLinDMA", l,2-dilinolenyloxy-N,N-dimethyl-3-aminopropane or "DLenDMA", N-dioleyl-N,N-dimethylammonium chloride or "DODAC", N,N-distearyl-N,N-dimethylarnrnonium bromide or "DDAB", N-(l,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide or "DMRIE", 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-l-(ci s,cis-9,12-octadecadienoxy)propane or "CLinDMA", 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy l-l-(cis,cis-9', l-2'-octadecadienoxy)propane or "CpLinDMA", N,N-dimethyl-3,4-dioleyloxybenzylamine or "DMOBA", 1 ,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane or "DOcarbDAP", 2,3-Dilinoleoyloxy-N,N-dimethylpropylamine or "DLinDAP", l,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane or "DLincarbDAP", l ,2-Dilinoleoylcarbamyl-3-dimethylaminopropane or "DLinCDAP", 2,2-dilinoleyl-4-dimethylaminomethyl-[l,3]-dioxolane or "DLin- -DMA", 2,2-dilinoleyl-4-dimethylaminoethyl-[l,3]-dioxolane or "DLin-K-XTC2-DMA", and 2-(2,2-di((9Z,12Z)-octadeca-9,l 2-dien- 1-yl)-l ,3-dioxolan-4-yl)-N,N-dimethylethanamine (DLin-KC2-DMA)) (see, WO 2010/042877; Semple et al., Nature Biotech. 28: 172-176 (2010)), or mixtures thereof. (Heyes, J., et al., J Controlled Release 107: 276-287 (2005); Morrissey, DV., et al., Nat. Biotechnol. 23(8): 1003-1007 (2005); PCT Publication WO2005/121348A1). In some

embodiments, one or more of the cationic lipids comprise at least one of an imidazole, dialkylamino, or guanidinium moiety.

[0145] In some embodiments, one or more cationic lipids may be chosen from XTC (2,2-Dilinoley1-4-dimethylaminoethy1-[1,3]-dioxolane), MC3 (((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate), ALNY-100 ((3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d] [1 ,3]dioxol-5-amine)), NC98-5 (4,7,13-tris(3-oxo-3-(undecylamino)propyl)-N1,N16-diundecyl-4,7,10,13-tetraazahexadecane-1,16-diamide), DODAP (1,2-dioleyl-3-dimethylammonium propane), HGT4003 (WO 2012/170889, the teachings of which are incorporated herein by reference in their entirety), ICE (WO 2011/068810, the teachings of which are incorporated herein by reference in their entirety), HGT5000 (U.S. Provisional Patent Application No. 61/617,468, the teachings of which are incorporated herein by reference in their entirety) or HGT5001 (cis or trans) (Provisional Patent Application No. 61/617,468), aminoalcohol lipidoids such as those disclosed in WO2010/053572, DOTAP (1,2-dioleyl-3-trimethylammonium propane), DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane), DLinDMA (Heyes, J.; Palmer, L.; Bremner, K.; MacLachlan, I. "Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids" J. Contr. Rel. 2005, 107, 276-287), DLin-KC2-DMA (Semple, S.C. et al. "Rational Design of Cationic Lipids for siRNA Delivery" Nature Biotech. 2010, 28, 172-176), C12-200 (Love, K.T. et al. "Lipid-like materials for low-dose in vivo gene silencing" PNAS 2010, 107, 1864-1869), N1GL, N2GL, V1GL and combinations thereof.

[0146] In some embodiments, the one or more cationic lipids are amino lipids. Amino lipids suitable for use in the invention include those described in WO2017180917, which is hereby incorporated by reference. Exemplary aminolipids in WO2017180917 include those described at paragraph [0744] such as DLin-MC3-DMA (MC3), (13Z,16Z)-N,N-dimethyl-3-nonyldocosa-13,16-dien-1-amine (L608), and Compound 18. Other amino lipids include Compound 2, Compound 23, Compound 27, Compound 10, and Compound 20. Further amino lipids suitable for use in the invention include those described in WO2017112865, which is hereby incorporated by reference. Exemplary amino lipids in WO2017112865 include a compound according to one of formulae (I), (Ial)-(Ia6), (lb), (II),

(IIa), (III), (IIIa), (IV), (17-1), (19-1), (19-11), and (20-1), and compounds of paragraphs [00185], [00201], [0276]. In some embodiments, cationic lipids suitable for use in the invention include those described in WO2016118725, which is hereby incorporated by reference. Exemplary cationic lipids in WO2016118725 include those such as KL22 and KL25. In some embodiments, cationic lipids suitable for use in the invention include those described in WO2016118724, which is hereby incorporated by reference. Exemplary cationic lipids in WO2016118725 include those such as KL10, 1 ,2-dilinoleyloxy-N,N-dimethylaminopropane (DLin-DMA), and KL25.

[0147] In some embodiments, cationic lipids constitute at least about 5%, 10%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 70% of the total lipids in a suitable lipid solution by weight or by molar. In some embodiments, cationic lipid(s) constitute(s) about 30-70 % (e.g., about 30-65%, about 30-60%, about 30-55%, about 30-50%, about 30-45%, about 30-40%, about 35-50%, about 35-45%, or about 35-40%) of the total lipid mixture by weight or by molar.

Non-cationic/Helper Lipids

[0148] As used herein, the phrase "non-cationic lipid" refers to any neutral, zwitterionic or anionic lipid. As used herein, the phrase "anionic lipid" refers to any of a number of lipid species that carry a net negative charge at a selected pH, such as physiological pH. Non-cationic lipids include, but are not limited to, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-l-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, l-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), or a mixture thereof.

[0149] In some embodiments, non-cationic lipids may constitute at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65% or 70% of the total lipids in a suitable lipid solution by weight or by molar. In some embodiments, non-cationic lipid(s) constitute(s) about 30-50 % (e.g., about 30-45%, about 30-40%, about 35-50%, about 35-45%, or about 35-40%) of the total lipids in a suitable lipid solution by weight or by molar.

Cholesterol-based Lipids

[0150] In some embodiments, a suitable lipid solution includes one or more cholesterol-based lipids. For example, suitable cholesterol-based cationic lipids include, for example, DC-Choi (N,N-dimethyl-N-ethylcarboxamidocholesterol), 1,4-bis(3-N-oleylamino-propyl)piperazine (Gao, et al. Biochem. Biophys. Res. Comm. 179, 280 (1991); Wolf et al. BioTechniques 23, 139 (1997); U.S. Pat. No. 5,744,335), or ICE. In some embodiments, cholesterol-based lipid(s) constitute(s) at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, or 70% of the total lipids in a suitable lipid solution by weight or by molar. In some embodiments, cholesterol-based lipid(s) constitute(s) about 30-50 % (e.g., about 30-45%, about 30-40%, about 35-50%, about 35-45%, or about 35-40%) of the total lipids in a suitable lipid solution by weight or by molar.

PEGylated Lipids

[0151] In some embodiments, a suitable lipid solution includes one or more PEGylated lipids. For example, the use of polyethylene glycol (PEG)-modified phospholipids and derivatized lipids such as derivatized ceramides (PEG-CER), including N-Octanoyl-Sphingosine-l-[Succinyl(Methoxy Polyethylene Glycol)-2000] (C8 PEG-2000 ceramide) is also contemplated by the present invention. Contemplated PEG-modified lipids include, but are not limited to, a polyethylene glycol chain of up to 2kDa, up to 3 kDa, up to 4kDa or up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length. In some embodiments, a PEG-modified or PEGylated lipid is PEGylated cholesterol

or PEG-2K. In some embodiments, particularly useful exchangeable lipids are PEG-ceramides having shorter acyl chains (e.g., C_{14} or C_{18}).

[0152] PEG-modified phospholipid and derivatized lipids may constitute at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, or 70% of the total lipids in a suitable lipid solution by weight or by molar. In some embodiments, PEGylated lipid lipid(s) constitute(s) about 30-50 % (e.g., about 30-45%, about 30-40%, about 35-50%, about 35-45%, or about 35-40%) of the total lipids in a suitable lipid solution by weight or by molar.

[0153] Various combinations of lipids, i.e., cationic lipids, non-cationic lipids, PEG-modified lipids and optionally cholesterol, that can used to prepare, and that are comprised in, pre-formed lipid nanoparticles are described in the literature and herein. For example, a suitable lipid solution may contain cKK-E12, DOPE, cholesterol, and DMG-PEG2K; C12-200, DOPE, cholesterol, and DMG-PEG2K; HGT5000, DOPE, cholesterol, and DMG-PEG2K; HGT5001, DOPE, cholesterol, and DMG-PEG2K; cKK-E12, DPPC, cholesterol, and DMG-PEG2K; C12-200, DPPC, cholesterol, and DMG-PEG2K; HGT5000, DPPC, chol, and DMG-PEG2K; HGT5001, DPPC, cholesterol, and DMG-PEG2K; or ICE, DOPE and DMG-PEG2K.Additional combinations of lipids are described in the art, e.g., U.S. Serial No. 62/420,421 (filed on November 10, 2016), U.S. Serial No. 62/421,021 (filed on November 11, 2016), U.S. Serial No. 62/464,327 (filed on February 27, 2017), and PCT Application entitled "Novel ICE-based Lipid Nanoparticle Formulation for Delivery of mRNA," filed on November 10, 2017, the disclosures of which are included here in their full scope by reference. The selection of cationic lipids, non-cationic lipids and/or PEG-modified lipids which comprise the lipid mixture as well as the relative molar ratio of such lipids to each other, is based upon the characteristics of the selected lipid(s) and the nature of the and the characteristics of the mRNA to be encapsulated. Additional considerations include, for example, the saturation of the alkyl chain, as well as the size, charge, pH, pKa, fusogenicity and toxicity of the selected lipid(s). Thus the molar ratios may be adjusted accordingly.

Pre-formed Nanoparticle Formation and Mixing Process

[0154] The present invention is based on the discovery of the surprisingly unexpected effect that mixing empty pre-formed lipid nanoparticles (i.e., lipid nanoparticles formed in the absence of mRNA) and mRNA has on the resulting encapsulated mRNA potency and efficacy.

[0155] In some previously disclosed processes, see U.S. patent Application Serial No. 14/790,562 entitled "Encapsulation of messenger RNA", filed July 2, 2015 and its provisional U.S. patent application Serial No. 62/020,163, filed July 2, 2014, the disclosure of which are hereby incorporated in their entirety, in some embodiments, the previous invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles by mixing an mRNA solution and a lipid solution, wherein the mRNA solution and/or the lipid solution are heated to a pre-determined temperature greater than ambient temperature prior to mixing, to form lipid nanoparticles that encapsulate mRNA.

[0156] The present invention relates to a novel method of formulating mRNA-containing lipid nanoparticles. In the present invention, a novel process for preparing a lipid nanoparticle containing mRNA has been identified, which involves combining pre-formed lipid nanoparticles with mRNA under conditions which, due to the order of addition of such components, the resultant formulated particles show improved potency and efficacy. The mixing of the components is achieved with pump systems which maintain the lipid/mRNA (N/P) ratio constant throughout the process and which also afford facile scale-up. In some embodiments, the process is performed at large scale. For example, in some embodiments, a composition according to the present invention contains at least about 1 mg, 5mg, 10 mg, 50 mg, 100 mg, 500 mg, or 1000 mg of encapsulated mRNA.

[0157] For certain cationic lipid nanoparticle formulations of mRNA, in order to achieve high encapsulation of mRNA, which is essential for protection and delivery of mRNA, the mRNA in citrate buffer has to be heated. In those processes or methods, the heating is required to occur before the formulation process (i.e. heating the separate components) as heating post-formulation (post-formation of nanoparticles) does not increase the encapsulation efficiency of the mRNA in the lipid nanoparticles. In contrast, in some

embodiments of the novel processes of the present invention, the order of heating of mRNA does not appear to affect the mRNA encapsulation percentage. In some embodiments, no heating (i.e., maintaining at ambient temperature) of one or more of the solution comprising the pre-formed lipid nanoparticles, the solution comprising the mRNA and the mixed solution comprising the lipid nanoparticle encapsulated mRNA is required to occur before or after the formulation process. This potentially provides a huge advantage for precisely scaling up, as controlled temperature change post-mixing is easier to achieve.

[0158] With this novel process, in some embodiments, encapsulating mRNA by using a step of mixing the mRNA with empty (i.e., empty of mRNA) pre-formed lipid nanoparticles (Process B) results in remarkably higher potency as compared to encapsulating mRNA by mixing the mRNA with just the lipid components (i.e., that are not pre-formed into lipid nanoparticles)(Process A). As described in the Examples below, for example in Tables 3 and 4, the potency of any mRNA encapsulated lipid nanoparticles tested is from more than 100% to more than 1000% more potent when prepared by Process B as compared to Process A.

[0159] In some embodiments, the empty (i.e., empty of mRNA) lipid nanoparticles without mRNA are formed by mixing a lipid solution containing dissolved lipids in a solvent, and an aqueous/buffer solution. In some embodiments, the solvent can be ethanol. In some embodiments, the aqueous solution can be a citrate buffer.

[0160] As used herein, the term "ambient temperature" refers to the temperature in a room, or the temperature which surrounds an object of interest (e.g., a pre-formed empty lipid nanoparticle solution, an mRNA solution, or a lipid nanoparticle solution containing mRNA) without heating or cooling. In some embodiments, the ambient temperature at which one or more of the solutions is maintained is or is less than about 35 °C, 30 °C, 25 °C, 20 °C, or 16 °C. In some embodiments, the ambient temperature at which one or more of the solutions is maintained ranges from about 15-35 °C, about 15-30 °C, about 15-25 °C, about 15-20 °C, about 20-35 °C, about 25-35 °C, about 30-35 °C, about 20-30 °C, about 25-30 °C or about 20-25 °C. In some embodiments, the ambient temperature at which one or more of the solutions is maintained is 20-25 °C.

[0161] Therefore, a pre-determined temperature greater than ambient temperature is typically greater than about 25 °C. In some embodiments, a pre-determined temperature suitable for the present invention is or is greater than about 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, or 70 °C. In some embodiments, a pre-determined temperature suitable for the present invention ranges from about 25-70 °C, about 30-70 °C, about 35-70 °C, about 40-70 °C, about 45-70 °C, about 50-70 °C, or about 60-70 °C. In particular embodiments, a pre-determined temperature suitable for the present invention is about 65 °C.

[0162] In some embodiments, the mRNA, or pre-formed empty (i.e., empty of mRNA) lipid nanoparticle solution, or both, may be heated to a pre-determined temperature above the ambient temperature prior to mixing. In some embodiments, the mRNA and the pre-formed empty lipid nanoparticle solution are heated to the pre-determined temperature separately prior to the mixing. In some embodiments, the mRNA and the pre-formed empty lipid nanoparticle solution are mixed at the ambient temperature but then heated to the pre-determined temperature after the mixing. In some embodiments, the pre-formed empty lipid nanoparticle solution is heated to the pre-determined temperature and mixed with mRNA at the ambient temperature. In some embodiments, the mRNA solution is heated to the pre-determined temperature and mixed with a pre-formed empty lipid nanoparticle solution at ambient temperature.

[0163] In some embodiments, the mRNA solution is heated to the pre-determined temperature by adding an mRNA stock solution that is at ambient temperature to a heated buffer solution to achieve the desired pre-determined temperature.

[0164] In some embodiments, the lipid solution containing dissolved lipids, or the aqueous/buffer solution, or both, may be heated to a pre-determined temperature above the ambient temperature prior to mixing. In some embodiments, the lipid solution containing dissolved lipids and the aqueous solution are heated to the pre-determined temperature separately prior to the mixing. In some embodiments, the lipid solution containing dissolved lipids and the aqueous solution are mixed at the ambient temperature but then heated to the pre-determined temperature after the mixing. In some embodiments, the lipid solution containing dissolved lipids is heated to the pre-determined temperature and mixed with an aqueous solution at the ambient temperature. In some embodiments, the aqueous solution is

heated to the pre-determined temperature and mixed with a lipid solution containing dissolved lipids at ambient temperature. In some embodiments, no heating of one or more of the solution comprising the pre-formed lipid nanoparticles, the solution comprising the mRNA and the mixed solution comprising the lipid nanoparticle encapsulated mRNA occurs before or after the formulation process.

[0165] In some embodiments, the lipid solution and an aqueous or buffer solution may be mixed using a pump. In some embodiments, an mRNA solution and a pre-formed empty lipid nanoparticle solution may be mixed using a pump. As the encapsulation procedure can occur on a wide range of scales, different types of pumps may be used to accommodate desired scale. It is however generally desired to use a pulse-less flow pumps. As used herein, a pulse-less flow pump refers to any pump that can establish a continuous flow with a stable flow rate. Types of suitable pumps may include, but are not limited to, gear pumps and centrifugal pumps. Exemplary gear pumps include, but are not limited to, Cole-Parmer or Diener gear pumps. Exemplary centrifugal pumps include, but are not limited to, those manufactured by Grainger or Cole-Parmer.

[0166] An mRNA solution and a pre-formed empty lipid nanoparticle solution may be mixed at various flow rates. Typically, the mRNA solution may be mixed at a rate greater than that of the lipid solution. For example, the mRNA solution may be mixed at a rate at least 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x, 15x, or 20x greater than the rate of the lipid solution.

[0167] Suitable flow rates for mixing may be determined based on the scales. In some embodiments, an mRNA solution is mixed at a flow rate ranging from about 40-400 ml/minute, 60-500 ml/minute, 70-600 ml/minute, 80-700 ml/minute, 90-800 ml/minute, 100-900 ml/minute, 110- 1000 ml/minute, 120-1100 ml/minute, 130- 1200 ml/minute, 140-1300 ml/minute, 150- 1400 ml/minute, 160- 1500 ml/minute, 170-1600 ml/minute, 180-1700 ml/minute, 150-250 ml/minute, 250-500 ml/minute, 500-1000 ml/minute, 1000-2000 ml/minute, 2000-3000 ml/minute, 3000-4000 ml/minute, or 4000-5000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow rate of about 200 ml/minute, about 500 ml/minute, about 1000 ml/minute, about 2000 ml/minute, about 3000 ml/minute, about 4000 ml/minute, or about 5000 ml/minute.

[0168] In some embodiments, a lipid solution or a pre-formed lipid nanoparticle solution is mixed at a flow rate ranging from about 25-75 ml/minute, 20-50 ml/minute, 25-75 ml/minute, 30-90 ml/minute, 40-100 ml/minute, 50-110 ml/minute, 75-200 ml/minute, 200-350 ml/minute, 350-500 ml/minute, 500-650 ml/minute, 650-850 ml/minute, or 850-1000 ml/minute. In some embodiments, the lipid solution is mixed at a flow rate of about 50 ml/minute, about 100 ml/minute, about 150 ml/minute, about 200 ml/minute, about 250 ml/minute, about 300 ml/minute, about 350 ml/minute, about 400 ml/minute, about 450 ml/minute, about 500 ml/minute, about 550 ml/minute, about 600 ml/minute, about 650 ml/minute, about 700 ml/minute, about 750 ml/minute, about 800 ml/minute, about 850 ml/minute, about 900 ml/minute, about 950 ml/minute, or about 1000 ml/minute.

[0169] Typically, in some embodiments, a lipid solution containing dissolved lipids, and an aqueous or buffer solution are mixed into a solution such that the lipids can form nanoparticles without mRNA (or empty pre-formed lipid nanoparticles). In some embodiments, an mRNA solution and a pre-formed lipid nanoparticle solution are mixed into a solution such that the mRNA becomes encapsulated in the lipid nanoparticle. Such a solution is also referred to as a formulation or encapsulation solution. A suitable formulation or encapsulation solution includes a solvent such as ethanol. For example, a suitable formulation or encapsulation solution includes about 10% ethanol, about 15% ethanol, about 20% ethanol, about 25% ethanol, about 30% ethanol, about 35% ethanol, or about 40% ethanol.

[0170] In some embodiments, a suitable formulation or encapsulation solution includes a solvent such as isopropyl alcohol. For example, a suitable formulation or encapsulation solution includes about 10% isopropyl alcohol, about 15% isopropyl alcohol, about 20% isopropyl alcohol, about 25% isopropyl alcohol, about 30% isopropyl alcohol, about 35% isopropyl alcohol, or about 40% isopropyl alcohol.

[0171] In some embodiments, a suitable formulation or encapsulation solution includes a solvent such as dimethyl sulfoxide. For example, a suitable formulation or encapsulation solution includes about 10% dimethyl sulfoxide, about 15% dimethyl sulfoxide, about 20% dimethyl sulfoxide, about 25% dimethyl sulfoxide, about 30% dimethyl sulfoxide, about 35% dimethyl sulfoxide, or about 40% dimethyl sulfoxide.

[0172] In some embodiments, a suitable formulation or encapsulation solution may also contain a buffering agent or salt. Exemplary buffering agent may include HEPES, ammonium sulfate, sodium bicarbonate, sodium citrate, sodium acetate, potassium phosphate and sodium phosphate. Exemplary salt may include sodium chloride, magnesium chloride, and potassium chloride.In some embodiments, an empty pre-formed lipid nanoparticle formulation used in making this novel nanoparticle formulation can be stably frozen in 10% trehalose solution.

[0173] In some embodiments, an empty (i.e., empty of mRNA) pre-formed lipid nanoparticle formulation used in making this novel nanoparticle formulation can be stably frozen in about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, or about 50% trehalose solution. In some embodiments, addition of mRNA to empty lipid nanoparticles can result in a final formulation that does not require any downstream purification or processing and can be stably stored in frozen form.

[0174] In some embodiments, ethanol, citrate buffer, and other destabilizing agents are absent during the addition of mRNA and hence the formulation does not require any further downstream processing. In some embodiments, the lipid nanoparticle formulation prepared by this novel process consists of pre-formed lipid nanoparticles in trehalose solution. The lack of destabilizing agents and the stability of trehelose solution increase the ease of scaling up the formulation and production of mRNA-encapsulated lipid nanoparticles.

Purification

[0175] In some embodiments, the empty pre-formed lipid nanoparticles or the lipid nanoparticles containing mRNA are purified and/or concentrated. Various purification methods may be used. In some embodiments, lipid nanoparticles are purified using Tangential Flow Filtration. Tangential flow filtration (TFF), also referred to as cross-flow filtration, is a type of filtration wherein the material to be filtered is passed tangentially *across* a filter rather than through it. In TFF, undesired permeate passes through the filter, while the desired retentate passes along the filter and is collected downstream. It is important

to note that the desired material is typically contained in the retentate in TFF, which is the opposite of what one normally encounters in traditional-dead end filtration.

[0176] Depending upon the material to be filtered, TFF is usually used for either microfiltration or ultrafiltration. Microfiltration is typically defined as instances where the filter has a pore size of between 0.05 μm and 1.0 μm, inclusive, while ultrafiltration typically involves filters with a pore size of less than 0.05 μm. Pore size also determines the nominal molecular weight limits (NMWL), also referred to as the molecular weight cut off (MWCO) for a particular filter, with microfiltration membranes typically having NMWLs of greater than 1,000 kilodaltons (kDa) and ultrafiltration filters having NMWLs of between 1 kDa and 1,000 kDa.

[0177] A principal advantage of tangential flow filtration is that non-permeable particles that may aggregate in and block the filter (sometimes referred to as "filter cake") during traditional "dead-end" filtration, are instead carried along the surface of the filter. This advantage allows tangential flow filtration to be widely used in industrial processes requiring continuous operation since down time is significantly reduced because filters do not generally need to be removed and cleaned.

[0178] Tangential flow filtration can be used for several purposes including concentration and diafiltration, among others. Concentration is a process whereby solvent is removed from a solution while solute molecules are retained. In order to effectively concentrate a sample, a membrane having a NMWL or MWCO that is substantially lower than the molecular weight of the solute molecules to be retained is used. Generally, one of skill may select a filter having a NMWL or MWCO of three to six times below the molecular weight of the target molecule(s).

[0179] Diafiltration is a fractionation process whereby small undesired particles are passed through a filter while larger desired nanoparticles are maintained in the retentate without changing the concentration of those nanoparticles in solution. Diafiltration is often used to remove salts or reaction buffers from a solution. Diafiltration may be either continuous or discontinuous. In continuous diafiltration, a diafiltration solution is added to the sample feed at the same rate that filtrate is generated. In discontinuous diafiltration, the

solution is first diluted and then concentrated back to the starting concentration. Discontinuous diafiltration may be repeated until a desired concentration of nanoparticles is reached.

[0180] Purified and/or concentrated lipid nanoparticles may be formulated in a desired buffer such as, for example, PBS.

Provided Nanoparticles Encapsulating mRNA

[0181] A process according to the present invention results in higher potency and efficacy thereby allowing for lower doses thereby shifting the therapeutic index in a positive direction. In some embodiments, the process according to the present invention results in homogeneous and small particle sizes (e.g., less than 150 nm), as well as significantly improved encapsulation efficiency and/or mRNA recovery rate as compared to a prior art process.

[0182] Thus, the present invention provides a composition comprising purified nanoparticles described herein. In some embodiments, majority of purified nanoparticles in a composition, i.e., greater than about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the purified nanoparticles, have a size of about 150 nm (e.g., about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, or about 80 nm). In some embodiments, substantially all of the purified nanoparticles have a size of about 150 nm (e.g., about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, or about 80 nm).

[0183] In addition, more homogeneous nanoparticles with narrow particle size range are achieved by a process of the present invention. For example, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified nanoparticles in a composition provided by the present invention have a size ranging from about 75-150 nm (e.g., about 75-145 nm, about 75-140 nm, about 75-135 nm, about 75-130 nm, about 75-125 nm, about 75-120 nm, about 75-115 nm, about 75-110 nm, about 75-105 nm, about 75-100

nm, about 75-95 nm, about 75-90 nm, or 75-85 nm). In some embodiments, substantially all of the purified nanoparticles have a size ranging from about 75-150 nm (e.g., about 75-145 nm, about 75-140 nm, about 75-135 nm, about 75-130 nm, about 75-125 nm, about 75-120 nm, about 75-115 nm, about 75-110 nm, about 75-105 nm, about 75-100 nm, about 75-95 nm, about 75-90 nm, or 75-85 nm).

[0184] In some embodiments, the dispersity, or measure of heterogeneity in size of molecules (PDI), of nanoparticles in a composition provided by the present invention is less than about 0.23 (e.g., less than about 0.23, 0.22, 0.21, 0.20, 0.19, 0.18, 0.17, 0.16, 0.15, 0.14, 0.13, 0.12, 0.11, 0.10, 0.09, or 0.08). In a particular embodiment, the PDI is less than about 0.16.

[0185] In some embodiments, greater than about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the purified lipid nanoparticles in a composition provided by the present invention encapsulate an mRNA within each individual particle. In some embodiments, substantially all of the purified lipid nanoparticles in a composition encapsulate an mRNA within each individual particle.

[0186] In some embodiments, a composition according to the present invention contains at least about 1 mg, 5 mg, 10 mg, 100 mg, 500 mg, or 1000 mg of encapsulated mRNA. In some embodiments, a process according to the present invention results in greater than about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% recovery of mRNA.

[0187] In some embodiments, a composition according to the present invention is formulated so as to administer doses to a subject. In some embodiments, a composition of mRNA lipid nanoparticles as described herein is formulated at a dose concentration of less than 1.0 mg/kg mRNA lipid nanoparticles (e.g., 0.6 mg/kg, 0.5 mg/kg, 0.3 mg/kg, 0.016 mg/kg. 0.05 mg/kg, and 0.016 mg/kg. In some embodiments, the dose is decreased due to the unexpected finding that lower doses yield high potency and efficacy. In some embodiments, the dose is decreased by about 70%, 65%, 60%, 55%, 50%, 45% or 40%.

[0188] In some embodiments, the potency of mRNA encapsulated lipid nanoparticles produced by Process B is from more than 100% (i.e., more than 200%, more than 300%,

more than 400%, more than 500%, more than 600%, more than 700%, more than 800%, or more than 900%) to more than 1000% more potent when prepared by Process B as compared to Process A.

EXAMPLES

[0189] While certain compounds, compositions and methods of the present invention have been described with specificity in accordance with certain embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

Lipid Materials

[0190] The formulations described in the following Examples, unless otherwise specified, contain a multi-component lipid mixture of varying ratios employing one or more cationic lipids, helper lipids (e.g., non-cationic lipids and/or cholesterol lipids) and PEGylated lipids designed to encapsulate various nucleic acid materials, as discussed previously.

Example 1. Lipid Nanoparticle Formulation Process A

[0191] This example illustrates an exemplary lipid nanoparticle formulation process for encapsulating mRNA. As used herein, Process A refers to a conventional method of encapsulating mRNA by mixing mRNA with a mixture of lipids, without first pre-forming the lipids into lipid nanoparticles. As compared to Process B described below, Process A does not involve pre-formation of lipid nanoparticles.

[0192] An exemplary formulation Process A is shown in **Figure 1**. In this process, in some embodiments, the ethanol lipid solution and the aqueous buffered solution of mRNA were prepared separately. A solution of mixture of lipids (cationic lipid, helper lipids, zwitterionic lipids, PEG lipids etc.) was prepared by dissolving lipids in ethanol. The mRNA solution was prepared by dissolving the mRNA in citrate buffer, resulting in mRNA at a concentration of 0.0833mg/ml in citrate buffer with a pH of 4.5. As shown in **Figure 1,** the mixtures were then both heated to 65 $^{\circ}$C prior to mixing. Then, these two solutions were

mixed using a pump system. In some instances, the two solutions were mixed using a gear pump system. In certain embodiments, the two solutions were mixing using a 'T' junction (or "Y" junction). The mixture was then purified by diafiltration with a TFF process. The resultant formulation concentrated and stored at 2-8 °C until further use.

Example 2. Lipid Nanoparticle Formulation Process B with Pre-Formed Lipid Nanoparticles

[0193] This example illustrates an exemplary Process B for encapsulating mRNA. As used herein, Process B refers to a process of encapsulating messenger RNA (mRNA) by mixing pre-formed lipid nanoparticles with mRNA. A range of different conditions, such as varying temperatures (i.e., heating or not heating the mixture), buffers, and concentrations, may be employed in Process B. The exemplary conditions described in this and other examples are for illustration purposes only.

[0194] An exemplary formulation Process B is shown in **Figure 2**. In this process, in some embodiments, lipids dissolved in ethanol and citrate buffer were mixed using a pump system. The instantaneous mixing of the two streams resulted in the formation of empty lipid nanoparticles, which was a self-assembly process. The resultant formulation mixture was empty lipid nanoparticles in citrate buffer containing alcohol. The formulation was then subjected to a TFF purification process wherein buffer exchange occurred. The resulting suspension of pre-formed empty lipid nanoparticles was then mixed with mRNA using a pump system. For certain cationic lipids, heating the solution post-mixing resulted in a higher percentage of lipid nanoparticles containing mRNA and a higher total yield of mRNA.

[0195] In addition, the effects of the presence of citrate buffer during the addition of mRNA in the Process B were studied. **Table 1** shows exemplary encapsulation efficiencies for lipid nanoparticle formulation Process B with and without citrate buffer (pH 4.5). Decreases in the encapsulation efficiency of mRNA were observed when citrate buffer was present during the mixing of pre-formed empty lipid nanoparticles and mRNA. In the presence of citrate buffer, the encapsulation efficiencies for the lipid nanoparticle formulation

Process B were less than 60%. The encapsulation efficiencies for the lipid nanoparticle formulation prepared by Process B without citrate buffer were around or above 90%.

Table 1 Encapsulation efficiencies for lipid nanoparticle formulation using Process B with and without citrate buffer.

Formulation	Lipid nanoparticle formulation – Process B in the absence of citrate buffer	Lipid nanoparticle formulation – Process B in the presence of citrate buffer
1	96	56
2	94	48
3	90	52
4	93	51

Example 3. In Vivo Activity of the Expressed hOTC in spf^{ash} Mice

[0196] This example illustrates that mRNA delivered via lipid nanoparticles produced by Process B were unexpectedly more effective than those produced by Process A.

[0197] In this example, OTC spf^{ash} mice were administered a single 0.5 mg/kg dose of hOTC mRNA encapsulated in lipid nanoparticles prepared by Process A or Process B. The liver tissues from these mice were analyzed 24 hours after administration for citrulline production. The formulations were first tested directly after mixing without storing, as well as tested after the mixture of the formulation was stored for a period of 2.5 months at -80 °C.

[0198] **Figure 3** depicts exemplary activity of expressed hOTC protein (in terms of citrulline production) in livers of OTC spf^{ash} mice 24 hours after a single 0.5 mg/kg dose of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or Process B.

[0199] Generally, the production of citrulline can be used to evaluate the activity of the expressed hOTC protein. As shown in **Figure 3**, citrulline activity due to expressed hOTC protein in OTC *spf^{ash}* mice liver was measured 24 hours after the single dose of the lipid nanoparticle mRNA formulation made by Process A and Process B, respectively. The graph (i) in **Figure 3** illustrates the citrulline activity due to expressed hOTC after the

delivery of the lipid nanoparticle mRNA formulation by Process A and Process B, respectively, right after the mixture of the formulation and without the storing time. Graph (ii) in **Figure 3** illustrates the citrulline activity due to expressed hOTC after the delivery of the lipid nanoparticle mRNA formulation by Process A and Process B, respectively, after the mixture of the formulation was stored for a period of 2.5 months at -80 °C.

[0200] The results shown in **Figure 3** indicate that the formulation prepared by Process B with pre-formed empty lipid nanoparticles resulted in about 3 times the citrulline activity of hOTC protein when compared to the formulation prepared by Process A. As evidenced by the similarity in the results shown in graphs (i) and (ii), formulations produced by both Process A and by Process B exhibited stability and functionality after extended storage at -80 °C.

Example 4. In Vivo Activity of the Expressed hOTC in spf^{ash} mice Under Different Process B Parameters

[0201] This example illustrates that lipid nanoparticles produced by Process B with different parameters, when delivered to *spf^{ash}* mice, lead to citrulline activity comparable to that seen in wild type mice.

[0202] In this example, OTC spf^{ash} mice were administered a single 0.5 mg/kg dose of hOTC mRNA encapsulated in lipid nanoparticles prepared by Process A or Process B. The liver tissues from these mice were analyzed 24 hours after administration for citrulline production. Four different lipid nanoparticle formulations were produced by Process B, each prepared using a different type of pump.

[0203] **Figure 4** depicts exemplary activity of expressed hOTC protein (in terms of citrulline production) in livers of OTC *spf^{ash}* mice 24 hours after a single 0.5 mg/kg dose of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or Process B. Lipid nanoparticle formulations made by Process B were prepared (1) using gear pumps, (2) using peristaltic pumps, (3) using peristaltic pumps at lower flow rates, and (4) using peristaltic pumps at different flow rates of mRNA and empty pre-formed lipid nanoparticles.

[0204] In some embodiments, the lipid nanoparticle formulations by Process B can be prepared under different process parameters, as shown in **Table 2**.

Table 2

Process B Formulation	Pump System	Flow Rate of Empty Lipid Nanoparticle Solution (mL/minute)	Flow Rate of mRNA Solution (mL/minute)
1	Gear Pump	50	50
2	Peristaltic Pump	50	50
3	Peristaltic Pump	10	10
4	Peristaltic Pump	50	10

[0205] In some embodiments, different formulations prepared by Process A and Process B formulations numbered 1-4 were tested *in vivo*.

[0206] Generally, the production of citrulline can be used to evaluate the activity of the expressed hOTC protein. As shown in **Figure 4**, citrulline activity of hOTC protein in OTC *spf*^{ash} mice liver was measured 24 hours after the single dose of a lipid nanoparticle mRNA formulation made by either Process A or Process B with different parameters.

[0207] As shown in **Figure 4**, the exemplary data demonstrate that when different lipid nanoparticle formulations made by Process B (numbered 1 - 4) were administered to *spf*^{ash} mice, the treatment led to surprisingly high levels of citrulline activity of hOTC protein that was comparable to that of wild type mice. At the same dosage level (0.5 mg/kg) of OTC mRNA, the lipid nanoparticle formulations prepared by Process B showed 2 - 4 times as much *in vivo* activity as the formulation prepared by Process A.

Example 5. In Vitro ASS1 Expression in 293T Cells

[0208] This example illustrates that the lipid nanoparticles prepared by Process B resulted in unexpectedly high protein expression in transfected cells.

[0209] **Figure 5** depicts exemplary human ASS1 protein expression in 293T cells 16 hours post-transfection with either naked hASS1 mRNA (with lipofectamine) or hASS1 mRNA-encapsulated lipid nanoparticles (without lipofectamine) produced by Process A or Process B.

[0210] In this example, 293T cells were transfected with ASS1 mRNA lipid nanoparticle formulations prepared by Process A or by Process B. Either 1 μg of ASS1 mRNA was transfected using lipofectamine or 10 μg of ASS1 mRNA encapsulated in lipid nanoparticle formulations were transfected per 10^6 cells for 24 hours. ASS1 protein expression was determined by ELISA.

[0211] As shown in **Figure 5**, the lipid nanoparticle formulation prepared by Process B lead to much higher levels of ASS1 protein expression than the formulation prepared by Process A. The levels of ASS1 protein expression resulting from transfection with lipid nanoparticles prepared by Process B was comparable to the levels resulting from transfection with an ASS1 mRNA-lipofectamine complex. The ASS1 protein level per 10^6 cells for the mRNA-lipofectamine complex, lipid nanoparticle formulation - Process A and lipid nanoparticle formulation - Process B was 12.43, 0.43 and 12.11 μg respectively. The ASS1 protein level resulting from transfection with a lipid nanoparticle formulation prepared by Process B was 28 times that from transfection with a lipid nanoparticle formulation prepared by Process A.

Example 6. In Vivo Expression of hCFTR in Rat Lungs

[0212] **Figure 6** shows exemplary immunohistochemical detection of hCFTR protein in rat lungs 24 hours after nebulization of hCFTR mRNA lipid nanoparticles prepared by Process B using different cationic lipids.

[0213] Male Sprague-Dawley rats were administered, via nebulization, lipid nanoparticle formulations containing hCFTR mRNA prepared by Process B. The lipid nanoparticle formulations were made using cKK-E12, ICE or Target 24 lipid as the cationic lipid. The fixed lung tissues from these rats were analyzed for the presence of hCFTR protein by immunohistochemical staining.

[0214] Protein was detected in both the bronchial epithelial cells as well as the alveolar regions. Positive (brown) staining was observed in all mRNA lipid nanoparticle test article groups, as compared to the lack of brown staining in the lungs of saline-treated control rats. Rats were dosed via nebulization with (i) saline, (ii) lipid nanoparticle formulation of cKK-E12 lipid prepared by Process B, (iii) lipid nanoparticle formulation of ICE lipid prepared by Process B, or (iv) lipid nanoparticle formulation of Target 24 lipid prepared by Process B.

Example 7. In Vivo Expression of hCFTR in Mice Lungs

[0215] **Figure** 7 shows exemplary immunohistochemical detection of hCFTR protein in mouse lungs 24 hours after nebulization of hCFTR mRNA lipid nanoparticles prepared by Process B.

[0216] In this example, C57BL mice were administered, via nebulization, lipid nanoparticles prepared by Process B that comprised cKK-E12 and contained hCFTR mRNA. The fixed lung tissues from these mice were analyzed for the presence of hCFTR protein by immunohistochemical staining.

[0217] Protein was detected in both the bronchial epithelial cells as well as the alveolar regions. Positive (brown) staining was observed for the mRNA lipid nanoparticle test article group, as compared to the lack of brown staining in the lungs of saline-treated control mice.

Example 8. In Vivo Expression of Firefly Luciferase Protein in Mice after Intravitreal Administration

[0218] This example illustrates exemplary methods of administering firefly luciferase (FFL) mRNA-loaded lipid nanoparticles produced by Process B and methods for analyzing firefly luciferase in target tissues *in vivo*.

[0219] **Figure 8** depicts bioluminescent imaging of wild type mice 24 hours after intravitreal administration of FFL mRNA encapsulated in lipid nanoparticles.

[0220] In this example, wild type mice were treated with lipid nanoparticles encapsulating mRNA encoding FFL produced by Process B via intravitreal administration. A solution containing 5 μg of FFL mRNA lipid nanoparticles was injected into the left eye of mice. Luminescence was monitored 24 hours after injection.

[0221] The results shown in **Figure 8** indicate that significant luminescence was observed, representing the successful production of active FFL protein in the eyes of these mice. Furthermore, sustained FFL activity was maintained for at least 24 hours.

Example 9. In Vivo Expression of Firefly Luciferase Protein in Mice After Topical Ocular Application

[0222] This example illustrates exemplary methods of administering firefly luciferase (FFL) mRNA-loaded lipid nanoparticles produced by Process B and methods for analyzing firefly luciferase in target tissues *in vivo*.

[0223] **Figure 9** depicts bioluminescent imaging of wild type mice 24 hours after topical application of eye drops containing FFL mRNA encapsulated in a lipid nanoparticle formulated with polyvinyl alcohol.

[0224] In this example, wild type mice were treated with lipid nanoparticles encapsulating mRNA encoding FFL produced by Process B via topical application (eye drops). A solution containing 5 μg of FFL mRNA lipid nanoparticles formulated with polyvinyl alcohol was applied to the right eye of mice. Luminescence was monitored 24 hours after application.

[0225] The results shown in **Figure 9** indicate that significant luminescence was observed, representing the successful production of active FFL protein in the eyes of these mice. Furthermore, sustained FFL activity was maintained for at least 24 hours.

Example 10. In Vivo Activity of PAH Expressed in Mice

[0226] In this example, phenylalanine hydroxylase (PAH) knockout (KO) mice were administered a single subcutaneous injection of 20.0 mg/kg hPAH lipid nanoparticles produced by Process B. Phenylalanine levels in the mice serum were measured 24 hours after administration.

[0227] **Figure 10** shows exemplary serum phenylalanine levels in PAH KO mice pre- and post-treatment with human PAH (hPAH) mRNA encapsulated in lipid nanoparticles produced by Process B. Serum samples were measured 24 hours after a single subcutaneous administration.

[0228] The mRNA-derived hPAH protein was shown to be enzymatically active, as demonstrated by measuring levels of serum phenylalanine reduction using a custom *ex vivo* activity assay. Generally, the reduction of serum phenylalanine can be used to evaluate the activity of the potency (i.e., expressed PAH protein) and the efficacy of the delivery method. As shown in **Figure 10**, exemplary serum phenylalanine levels in PAH KO mice were measured before and 24 hours after the single dose of the lipid nanoparticle encapsulating hPAH mRNA formulation prepared by Process B that was delivered subcutaneously. As a comparison, serum phenylalanine levels in saline treated PAH KO mice were also measured.

[0229] The results shown in **Figure 10** indicate that the subcutaneously injected lipid nanoparticle hPAH mRNA formulation resulted in significant phenylalanine level reduction. There was not a significant difference in phenylalanine levels in saline-treated PAH KO mice before and after the dose.

Example 11. In Vivo Activity of OTC Expressed in Mice

[0230] This example shows a comparison of levels of OTC protein activity in the livers of saline treated OTC KO *spf*^{ash} mice and OTC KO *spf*^{ash} mice treated with subcutaneous administration of hOTC mRNA–loaded lipid nanoparticles made by Process B.

[0231] As shown in **Figure 11**, exemplary citrulline production as a result of expressed hOTC protein in the livers of OTC KO *spf*^{ash} mice was measured 24 hours after the single subcutaneous dose of the lipid nanoparticle encapsulating hOTC mRNA formulation made by Process B. In addition, as a comparison, citrulline production in livers of OTC KO *spf*^{ash} mice was measured after saline was injected.

[0232] The results shown in **Figure 11** indicate that the subcutaneously injected lipid nanoparticle hOTC mRNA formulation made by Process B resulted in significant activity of the expressed hOTC protein 24 hours post-dose compared with saline treated diseased mice.

Example 12. In Vivo Expression of ASS1 in Mice

[0233] **Figure 12** depicts exemplary human ASS1 protein levels measured in ASS1 KO mice liver 24 hours after a single subcutaneous administration of lipid nanoparticle formulation encapsulating hASS1 mRNA made by Process B.

[0234] Generally, the expressed hASS1 protein levels can be used to evaluate the efficiency of the delivery method. As shown in **Figure 12**, exemplary hASS1 protein level in ASS1 KO mice was measured 24 hours after the single subcutaneous dose of the lipid nanoparticle encapsulating hASS1 mRNA formulation made by Process B. In addition, as a comparison, hASS1 protein level in ASS1 KO mice treated with saline was also measured.

[0235] The results shown in **Figure 12** indicate that subcutaneously injected hASS1 mRNA lipid nanoparticle formulation made by Process B resulted in significant hASS1 protein level in hASS1 KO mice 24 hours post-dose when compared with saline-treated hASS1 KO mice.

Example 13. In Vivo Expression of hEPO in Mice Via Various Routes of Administration

[0236] This example shows a comparison of expressed human EPO (hEPO) in wild type mice after administration of hEPO mRNA encapsulated in lipid nanoparticles made by Process B by various routes. This example further illustrates a comparison of potency of mRNA delivered via lipid nanoparticles produced by Process A and Process B for intradermal and intramuscular administration at various dose levels. It is shown that mRNA delivered via lipid nanoparticles produced by process B were substantially more potent than those produced by Process A at all dosages and time points, whether delivered by intradermal or intramuscular routes of administration assessed.

[0237] In this example, wild type mice were administered via intradermal, subcutaneous, or intramuscular routes a single dose at varying concentrations (i.e., 1 μg, 10 μg, or 50 μg) of hEPO mRNA encapsulated lipid nanoparticles produced by Process B. Serum levels of hEPO protein were measured 6 hours and 24 hours after administration. Additionally, wild type mice were administered via intradermal or intramuscular routes a single dose at varying concentrations (i.e., 1 μg, 10 μg, or 50 μg) of hEPO mRNA encapsulated lipid nanoparticles produced by Process A or Process B. Serum levels of hEPO protein were measured 6 hours and 24 hours after administration.

[0238] **Figure 13** depicts exemplary hEPO protein levels measured in the serum of treated mice 6 hours and 24 hours after a single administration of hEPO mRNA formulation made by Process B. The routes compared were administration by intradermal, subcutaneous or intramuscular injection.

[0239] The levels of hEPO protein in the serum of mice after treatment can be used to evaluate the potency of mRNA via the different delivery methods. As shown in **Figure 13**, exemplary hEPO protein levels protein in mice sera were evaluated by ELISA 6 hours and 24 hours after the single dose of the hEPO mRNA lipid nanoparticle formulation made by Process B at 1 μg, 10 μg and 50 μg. In addition, hEPO protein levels from intradermal, subcutaneous and intramuscular routes of administration were compared.

[0240] The results shown in **Figure 13** indicate that the hEPO mRNA lipid nanoparticle formulation intramuscularly injected generally resulted in highest levels of

hEPO protein when compared to the intradermal and subcutaneous routes. At 6 hours post-dose, hEPO protein levels were slightly higher from subcutaneous administration than from intradermal administration.

Comparison of mRNA lipid nanoparticles produced by Process A and Process B for intradermal and intramuscular administration at various dose levels

[0241] **Figure 14** depicts a comparison of hEPO protein levels measured in the serum of treated mice 6 hours and 24 hours after a single intradermal dose of hEPO mRNA encapsulated in lipid nanoparticle formulation made by Process A or Process B. **Figure 14** indicates that, at all doses, the formulation prepared by Process B resulted in about 2-4 times higher hEPO protein level expression as compared to the formulation prepared by Process A.

[0242] **Figure 15** depicts a comparison of hEPO protein levels measured in the serum of treated mice 6 hours and 24 hours after a single intramuscular dose of hEPO mRNA encapsulated in lipid nanoparticle formulation made by Process A or Process B. **Figure 15** indicates that, at all doses, the formulation prepared by Process B resulted in about 2-4 times higher hEPO protein level expression as compared to the formulation prepared by Process A.

[0243] As shown in **Figure 14** and **Figure 15**, hEPO protein levels protein in mice sera were evaluated by ELISA 6 hours and 24 hours after the single dose of the hEPO mRNA lipid nanoparticle formulation made by Process A or Process B at 1 μg, 10 μg and 50 μg via intradermal and intramuscular administration, respectively. The results show substantially higher potency of the mRNA encapsulated lipid nanoparticles produced by Process B. It also was observed that higher potency of the Process B formulation was associated with various cells of the integumentary system (i.e., myocytes, fibroblasts, macrophages, adipocytes, etc.).

Example 14. Protein Expression from mRNA Lipid Nanoparticles in an Animal Model

[0244] This example illustrates significantly improved *in vivo* protein expression with mRNA delivered via lipid nanoparticles produced by Process B as compared to Process A, across a range of dose levels.

[0245] In this study, male *spf*^{ash} mice were treated with hOTC mRNA lipid nanoparticles produced by Process A or by Process B, in each case at four different dose levels (0.50 mg/kg, 0.16 mg/kg, 0.05 mg/kg, and 0.016 mg/kg). The test articles used throughout the study were the same except for the noted differences in the lipid nanoparticle production process (Process A versus Process B) and dose.

[0246] The test articles were administered as a single dose via tail vein injection. At 24 hours post administration mice were presented with an ammonia challenge wherein a bolus injection of ammonium chloride (5mmol/kg NH_4Cl) was administered intraperitoneally. Blood was collected 40 minutes after the NH_4Cl challenge by collecting aliquots of whole blood into lithium heparin plasma tubes, which were processed to plasma and plasma ammonia was analyzed using an IDEXX Catalyst Dx analyzer. Then the animals were sacrificed, and their livers were harvested and assessed for hOTC expression using sandwich ELISA.

[0247] **Figure 16** depicts a schematic of the ammonia challenge portion of the study, which was performed to represent a hyperammonemic episode that a patient suffering from OTC deficiency could experience.

[0248] **Figure 17** shows the plasma ammonia levels of each of the ammonia-challenged mice, particularly the wild-type mice having normal murine OTC (WT), *spf*^{ash} mice that received no hOTC mRNA lipid nanoparticles (KO), and *spf*^{ash} mice that received a single dose of 0.5, 0.16, 0.05 or 0.016 mg/kg mRNA lipid nanoparticles produced by Process B. As shown by the figure, a statistically significant protection of the model hyperammonemic episode was achieved at the 0.5 mg/kg and at the 0.16 mg/kg doses of the hOTC mRNA lipid nanoparticles produced by Process B, as compared to the marked elevations in plasma ammonia under identical conditions for the *spf*^{ash} mice that received no hOTC mRNA lipid nanoparticles (KO). This data illustrates that mRNA lipid nanoparticles produced by Process B and administered at doses of 0.5 mg/kg and at 0.16 mg/kg was effective for at least 24 hours following administration in protecting against an ammonium chloride challenge.

[0249] **Figure 18** and **Table 3** show the hOTC protein levels from the livers of the animals sacrificed at 24 hours post administration of the hOTC mRNA lipid nanoparticles, as measured by sandwich ELISA. As these results show, the hOTC protein expressed from livers of mice treated with the mRNA lipid nanoparticles prepared by Process B was nearly 1000% (i.e., ten times) higher than that from livers of mice treated with the same mRNA lipid nanoparticles prepared by Process A. **Table 3** provides the particular amounts of hOTC protein (as a percent of total protein) expressed from livers of mice treated with the mRNA lipid nanoparticles prepared by Process A and by Process B for all doses 24 hours following administration.

Table 3 *In vivo* hOTC protein expression measured 24 hours following administration of different doses (as shown) of hOTC mRNA lipid nanoparticles formulated by Process A or Process B.

Dose (mg/kg)	µg hOTC/mg protein Process A	µg hOTC/mg protein Process B	Avg. increase in hOTC expression from Process A to Process B
0.5	4.26 ± 1.90	35.12 ± 14.94	724% (8.2x)
0.16	0.73 ± 0.46	6.90 ± 1.61	845% (9.35x)
0.05	0.18 ± 0.19	2.01 ± 1.19	1016% (11.04x)
0.016	0.012 ± 0.006	0.126 ± 0.077	950% (10.01x)
		Average across all doses	884% (9.65x)

[0250] As shown by **Table 3**, the amount of hOTC protein expressed (as a percent of total protein) from livers of mice treated with the mRNA lipid nanoparticles prepared by Process B exceeded by more than about 700% (about 8x) and by up to about 1000% (about 11x) the amount of hOTC protein expressed (as a percent of total protein) from livers of mice treated with the mRNA lipid nanoparticles prepared by Process A, across all dosages at 24 hours following administration. The overall average increase in the amount of hOTC protein expressed (as a percent of total protein) from livers of mice treated with the mRNA lipid nanoparticles prepared by Process B versus by Process A was 884% (9.65x), across all dosages at 24 hours following administration. This data illustrates that mRNA lipid

nanoparticles produced by Process B were significantly more potent than the same mRNA lipid nanoparticles produced by Process A, at all doses, at 24 hours following administration.

[0251] **Figure 19** shows a comparison of hOTC protein amount in liver tissue of OTC *spf*^{ash} mice 24 hours after a single intravenous injection of hOTC mRNA at various doses (i.e., 0.5 mg/kg, 0.16 mg/kg, 0.05 mg/kg, and 0.016 mg/kg) encapsulated in lipid nanoparticle formulations made by Process A or Process B. As can be seen in the figure, the formulation doses produced by Process B resulted in more copies of hOTC mRNA per mg tissue than did the formulation produced by Process A. **Figure 20** shows a comparison of hOTC protein amount in RNA tested of OTC *spf*^{ash} mice 24 hours after a single intravenous injection of hOTC mRNA at various doses (i.e., 0.5 mg/kg, 0.16 mg/kg, 0.05 mg/kg, and 0.016 mg/kg) encapsulated in lipid nanoparticle formulations made by Process A or Process B. As can be seen in the figure, the formulation doses produced by Process B resulted in more copies of hOTC mRNA per μg RNA tested than did the formulation produced by Process A.

Example 15. Duration of Activity by Protein Expressed from mRNA Lipid Nanoparticles in an Animal Model

[0252] In this example, the activity of an exemplary protein expressed *in vivo* from an mRNA lipid nanoparticle persisted for an extended duration of at least 15 days.

[0253] In this study, male *spf*^{ash} mice were administered via single intravenous tail vein injection a dose of 1.0 mg/kg hOTC mRNA lipid nanoparticles produced by Process B. At each timepoint of 24 hours (Day 2), 48 hours (Day 3), 72 hours (Day 4), 96 hours (Day 5), 8 days (Day 8), 11 days (Day 11), and 15 days (Day 15) following administration a cohort of the mice were removed.

[0254] For each removed cohort, animals were subjected to an ammonia challenge after which blood was collected for plasma ammonia (μmol/L) measurements. Then the animals were sacrificed and citrulline (μmol citrulline/hr/mg of total protein) and urinary orotic acid (μmol/mmol creatinine) were measured.

[0255] For the ammonia challenge and plasma ammonia measurements, see **Figure 16** for a general schematic and Example 14 for a description of this test.

[0256] For the citrulline measurements, mouse liver homogenate was prepared and diluted in 1x DPBS then added into UltraPure water. Citrulline standard was added in predetermined amounts to serve as an internal reference. A reaction mix containing carbamoyl phosphate, ornithine and triethanolamine was added and the reaction was allowed to proceed at 37°C for 30 minutes. The reaction was stopped with a mix of phosphoric and sulfuric acid, and diacetylmonoxime was added. The sample was incubated at 85 degrees Celsius for 30 minutes, cooled briefly, and read at 490nm to quantify the citrulline against the citrulline standard.

[0257] For the urinary orotic acid measurements, orotic acid quantification from animal urine samples was performed via Ultra Performance Liquid Chromatography (UPLC) using an ion exchange column. Briefly, urine samples were diluted two-fold using RNase-free water and a portion was loaded onto a ThermoScientific 100x column. The mobile phase comprising acetonitrile and 25mM ammonium acetate afforded separation and quantification of orotic acid with detection based on absorbance at 280 nm.

[0258] **Figure 21** shows the plasma ammonia in animals 40 minutes after being subjected to an ammonia challenge, for each of wildtype mice (WT), untreated *spf*^{ash} mice (Untreated), and *spf*^{ash} mice at 24 hours (Day 2), 48 hours (Day 3), 72 hours (Day 4), 96 hours (Day 5), 8 days (Day 8), 11 days (Day 11), and 15 days (Day 15) following administration of 1.0 mg/kg hOTC mRNA lipid nanoparticles produced by Process B. The dashed line represents the average plasma ammonia level of the wild-type control group (WT). As can be seen by the results depicted in the figure, a single dose of the hOTC mRNA lipid nanoparticles provides significant protection against hyperammonemia for at least 15 days. Specifically, the plasma ammonia levels post challenge are comparable to wild type levels (WT) or far less than untreated levels (Untreated) at all the time points assessed out to 15 days.

[0259] **Figure 22** shows the hOTC protein activity as measured by citrulline production in each of wildtype mice (WT), untreated *spf*^{ash} mice (Untreated), and *spf*^{ash} mice at 24 hours (Day 2), 48 hours (Day 3), 72 hours (Day 4), 96 hours (Day 5), 8 days (Day 8), 11 days (Day 11), and 15 days (Day 15) following administration of 1.0 mg/kg hOTC mRNA lipid nanoparticles produced by Process B. As can be seen by the results depicted in the

figure, a single dose of hOTC mRNA lipid nanoparticles provides a resulting citrulline level that exceeds or is comparable to the wild type control (WT) and far exceeds the untreated control (Untreated) at all the time points assessed out to 15 days.

[0260] **Figure 23** shows the hOTC protein activity as measured by maintained low levels of urinary orotic acid production in each of untreated *spf*^{ash} mice (Untreated), *spf*^{ash} mice at 24 hours (Day 2), 48 hours (Day 3), 72 hours (Day 4), 96 hours (Day 5), 8 days (Day 8), 11 days (Day 11), and 15 days (Day 15) following administration of 1.0 mg/kg hOTC mRNA lipid nanoparticles produced by Process B, and untreated wildtype mice (Untreated C57BL/6). As can be seen by the results depicted in the figure, a single dose of hOTC mRNA lipid nanoparticles provides a resulting low level of urinary orotic acid that is less than or is comparable to the wild type control (Untreated C57BL/6) and is far less than the untreated *spf*^{ash} mice (Untreated) at all the time points assessed out to 15 days.

[0261] Taken together, the data in this example shows that a single intravenous administration of an exemplary mRNA lipid nanoparticle produced by Process B yields active protein that is active across several measures for at least 15 days.

Example 16. In Vivo Activity of the Expressed hOTC in spf^{ash} Mice at Various Doses

[0262] This example illustrates that hOTC mRNA, at three different dose levels (1.0 mg/kg, 0.6 mg/kg, and 0.3 mg/kg), delivered via lipid nanoparticles produced by Process B were unexpectedly more potent than those produced by Process A at each dose evaluated.

[0263] In this example, OTC spf^{ash} mice were administered a single intravenous dose (at varying concentrations, i.e., 1 mg/kg, 0.6 mg/kg, or 0.3 mg/kg) of hOTC mRNA encapsulated in lipid nanoparticles produced by Process A or Process B. The liver tissues from these mice were analyzed 24 hours after administration for citrulline production.

[0264] **Figure 24** depicts exemplary activity of expressed hOTC protein (in terms of citrulline production) in livers of OTC spf^{ash} mice 24 hours after a single intravenous dose of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or Process B. The hOTC mRNA was administered at different dosing levels of 1.0 mg/kg, 0.6 mg/kg, and 0.3 mg/kg.

[0265] Generally, the production of citrulline can be used to evaluate the activity of the expressed hOTC protein. As shown in **Figure 24**, citrulline activity due to expressed hOTC protein in OTC *spf*^{ash} mice liver was measured 24 hours after the single dose of the lipid nanoparticle mRNA formulation made by Process A and Process B, respectively, at various dose levels. The graph in **Figure 24** illustrates the citrulline activity due to expressed hOTC after the delivery of the lipid nanoparticle mRNA formulation by Process A and Process B, formulated per the processes described above.

[0266] The results shown in **Figure 24** indicate that the formulation prepared by Process B with pre-formed empty lipid nanoparticles resulted in higher citrulline activity of hOTC protein when compared to the formulation prepared by Process A.

[0267] **Figure 25** depicts the immunohistochemical detection of hOTC protein in the mice livers by Western blot images after the single intravenous dose of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or Process B at various dosing levels (i.e., 1.0 mg/kg, 0.6 mg/kg, and 0.3 mg/kg). As shown in the Figure, at all three doses, the hOTC protein expressed was higher for the group dosed by lipid nanoparticle formulation made by Process B as compared to Process A, as evidenced by intensity of the bands.

Example 17. In Vivo Activity of the Expressed hOTC in spf^{ash} Mice

[0268] This example illustrates that hOTC mRNA delivered via lipid nanoparticles produced by Process B were unexpectedly more effective than those produced by Process A.

[0269] Figure **26** depicts exemplary activity of expressed hOTC protein (in terms of citrulline production) in livers of OTC *spf*^{ash} mice 24 hours after a single intravenous 0.5 mg/kg dose of hOTC mRNA encapsulated in lipid nanoparticle formulations made by Process A or Process B.

[0270] Generally, the production of citrulline can be used to evaluate the activity of the expressed hOTC protein. As shown in **Figure 26**, citrulline activity due to expressed hOTC protein in OTC *spf*^{ash} mice liver was measured 24 hours after the dose was

administered and the results indicate that, at equal doses, the formulation prepared by Process B resulted in higher citrulline activity of hOTC protein as compared to the formulation prepared by Process A.

[0271] **Figure 27(a)-(d)** shows the immunohistochemical detection of hOTC protein in mouse livers 24 hours after dosing of hOTC mRNA lipid nanoparticles prepared by Process A or Process B via immunohistochemical staining. As can be seen in the Figure, the staining of hOTC protein is stronger for the mice group dosed with LMP formulation prepared by Process B (**Figure 27(a)-(b)**), as compared to Process A (**Figure 27(c)-(d)**). The results shown in **Figure 27(a)-(d)** agree with the higher citrulline production of the dose of the formulation prepared by Process B as compared to Process A, as depicted in **Figure 26**.

Example 18. In vivo Expression of mRNA Lipid Nanoparticle Formulations Prepared by Process B and Process A Using Different Cationic Lipids

[0272] This example illustrates that EPO mRNA delivered via lipid nanoparticles (consisting of a variety of different cationic lipids) produced by Process B were unexpectedly more effective than those produced by Process A.

[0273] In this study, male CD1 mice were administered on Day 1 a single intravenous tail vein injection at a dose of 1.0 mg/kg hEPO mRNA lipid nanoparticles each prepared using one of five different cationic lipids and produced by Process A or by Process B (as described earlier).

[0274] **Table 4** provides the particular hEPO protein expression levels as measured in the serum of the animals sacrificed at 6 hours post administration of the hEPO mRNA lipid nanoparticles each prepared using one of five different cationic lipids and produced by Process A or by Process B, as measured by ELISA. As these results show, the hEPO protein expressed, as measured in the serum of the mice, from the mRNA lipid nanoparticles prepared by Process B was substantially higher than the same mRNA lipid nanoparticles prepared by Process A, across all the different cationic lipids evaluated. The percent increase ranged from 133% to 603%, with a consistent increase of greater than 100% potency observed across the 5 different lipids tested in the study.

[0275] **Figure 28** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using HGT 5001 as the cationic lipid. **Figure 29** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using ICE as the cationic lipid. **Figure 30** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using cKK-E12 as the cationic lipid. **Figure 31** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using C12-200 as the cationic lipid. **Figure 32** depicts hEPO protein expression after the delivery of the lipid nanoparticle mRNA formulation produced by Process A and Process B, formulated using HGT 4003 as the cationic lipid. As the results in each of these **Figures 28-32** graphs show, the hEPO protein expressed, as measured in the serum of the mice, from the mRNA lipid nanoparticles prepared by Process B was substantially higher than the same mRNA lipid nanoparticles prepared by Process A, across all five different cationic lipids evaluated.

Table 4. *In vivo* Human EPO protein expression measured in mouse serum 6 hours following administration of hEPO mRNA lipid nanoparticles (across a variety of cationic lipids) formulated by Process A or Process B.

	Human EPO protein measured in mouse serum 6 hours post dosing (µg/mL)		
Lipid	**Process A**	**Process B**	**% increase**
HGT 5001	1.33 ± 0.56	8.83 ± 4.63	565 %
ICE	0.0048 ± 0.002	0.0181 ± 0.008	279 %
cKK-E12	16.82 ± 5.92	118.4 ± 21.3	603 %
C12-200	25.7 ± 4.9	71.3 ± 11.3	176 %
HGT 4003	0.073 ± 0.008	0.172 ± 0.027	133 %

[0276] **Table 5** depicts structural details of hEPO lipid nanoparticles prepared by Process A or Process B, using various cationic lipids. In particular, **Table 5** depicts nanoparticle size (nm) and PdI of hEPO lipid nanoparticles when different cationic lipids are employed, as prepared by Process A or Process B. As shown in the Table, the nanoparticle sizes of hEPO mRNA lipid nanoparticles prepared by Process B were between about 90 nm

and 150 nm across all nanoparticles prepared using the five different cationic lipids evaluated whereas those prepared by Process A were between about 75 nm and 95 nm across all nanoparticles prepared using the five cationic lipids evaluated.

Table 5.

Group #	Cationic Lipid	Concentration (mg/ml)	Type	Size (nm)	PdI
1	N/A (Saline)	N/A	N/A	N/A	N/A
2	HGT5001	0.2	Process A	85.21	0.26
3	HGT 5001	0.2	Process B	128.3	0.16
4	ICE	0.2	Process A	79.02	0.27
5	ICE	0.2	Process B	147.4	0.23
6	cKK-E12	0.2	Process A	91.55	0.19
7	cKK-E12	0.2	Process B	126	0.16
8	C12-200	0.2	Process A	77.12	0.204
9	C12-200	0.2	Process B	116	0.18
10	HGT4003	0.2	Process A	89.31	0.2
11	HGT4003	0.2	Process B	91.05	0.16

[0277] Taken together, the data in this example shows that there is a substantial increase in potency for mRNA lipid nanoparticles produced by Process B as compared to by Process A, across lipid nanoparticles comprising various different lipid components.

EQUIVALENTS

[0278] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention

described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the following claims:

CLAIMS

We claim:

1. A process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising:

mixing a solution comprising pre-formed lipid nanoparticles and mRNA such that lipid nanoparticles encapsulating mRNA are formed.

2. The process of claim 1, wherein the solution comprising pre-formed lipid nanoparticles and mRNA comprises less than 10mM citrate.

3. The process of claim 1, wherein the solution comprising pre-formed lipid nanoparticles and mRNA comprises less than 25% non-aqueous solvent.

4. The process of claim 1, further comprising heating the lipid nanoparticles and mRNA to a temperature greater than ambient temperature after the mixing.

5. The process of claim 1, wherein the mRNA and/or the pre-formed lipid nanoparticles are heated to a temperature greater than ambient temperature prior to the mixing.

6. The process of claims 4 or 5, wherein the temperature is or is greater than about 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, or 70 °C.

7. The process of any one of claims 4-6, wherein the temperature ranges from about 25-70 °C, about 30-70 °C, about 35-70 °C, about 40-70 °C, about 45-70 °C, about 50-70 °C, or about 60-70 °C.

8. The process of any one of claims 4-7, wherein the temperature is about 65 °C.

9. The process of any one of the preceding claims, wherein the pre-formed lipid nanoparticles are formed by mixing lipids dissolved in ethanol with an aqueous solution.

10. The process of claim 9, wherein the lipids comprise one or more cationic lipids, one or more helper lipids, one or more cholesterol-based lipids and PEG lipids.

11. The process of claim 10, wherein the one or more cationic lipids are selected from the group consisting of cKK-E12, OF-02, C12-200, MC3, DLinDMA, DLinkC2DMA, ICE (Imidazol-based), HGT5000, HGT5001, HGT4003, DODAC, DDAB, DMRIE, DOSPA, DOGS, DODAP, DODMA and DMDMA, DODAC, DLenDMA, DMRIE, CLinDMA, CpLinDMA, DMOBA, DOcarbDAP, DLinDAP, DLincarbDAP, DLinCDAP, KLin-K-DMA, DLin-K-XTC2-DMA, 3-(4-(bis(2-hydroxydodecyl)amino)butyl)-6-(4-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)butyl)-1,4-dioxane-2,5-dione (Target 23), 3-(5-(bis(2-hydroxydodecyl)amino)pentan-2-yl)-6-(5-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)pentan-2-yl)-1,4-dioxane-2,5-dione (Target 24), and combinations thereof.

12. The process of claim 10, wherein the one or more cationic lipids comprise Target 24.

13. The process of claim 10, wherein the one or more cationic lipids comprise ICE.

14. The process of claim 10, wherein the one or more cationic lipids comprise cKK-E12.

15. The process of claim 10, wherein the one or more non-cationic lipids are selected from DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleyl-sn-glycero-3-phosphoethanolamine), DOPC (1,2-dioleyl-sn-glycero-3-phosphotidylcholine) DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), DOPG (1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)).

16. The process of claim 10, wherein the one or more PEG-modified lipids comprise a poly(ethylene) glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length.

17. The process of any one of the preceding claims, wherein the pre-formed lipid nanoparticles are purified by a Tangential Flow Filtration (TFF) process.

18. The process of claim 17, wherein greater than about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the purified nanoparticles have a size ranging from 75-150 nm.

19. The process of any one of claims 17 or 18, wherein substantially all of the purified nanoparticles have a size ranging from 75-150 nm.

20. The process of any one of claims 17-19, wherein greater than about 70% , 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified nanoparticles have a size ranging from 50-80 nm.

21. The process of any one of claim 15-18, wherein substantially all of the purified nanoparticles have a size ranging from 75-150 nm.

22. The process of any one of the preceding claims, wherein the process results in an encapsulation rate of greater than about 90%, 95%, 96%, 97%, 98%, or 99%.

23. The process of any one of the preceding claims, wherein the process results in greater than about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% recovery of mRNA.

24. The process of any one of the preceding claims, wherein the pre-formed lipid nanoparticles and mRNA are mixed using a pump system.

25. The process of claim 22, wherein the pump system comprises a pulse-less flow pump.

26. The process of claim 23, wherein the pump is a gear pump.

27. The process of any one of the preceding claims, wherein the solution comprising pre-formed lipid nanoparticles is mixed at a flow rate ranging from about 25-75 ml/minute, about 75-200 ml/minute, about 200-350 ml/minute, about 350-500 ml/minute, about 500-650 ml/minute, about 650-850 ml/minute, or about 850-1000 ml/minute.

28. The process of any one of the preceding claims, wherein the solution comprising pre-formed lipid nanoparticles is mixed at a flow rate of about 50 ml/minute, about 100 ml/minute, about 150 ml/minute, about 200 ml/minute, about 250 ml/minute, about 300 ml/minute, about 350 ml/minute, about 400 ml/minute, about 450 ml/minute, about 500 ml/minute, about 550 ml/minute, about 600 ml/minute, about 650 ml/minute, about 700 ml/minute, about 750 ml/minute, about 800 ml/minute, about 850 ml/minute, about 900 ml/minute, about 950 ml/minute, or about 1000 ml/minute.

29. The process of any one of the preceding claims, wherein the mRNA is mixed in a solution at a flow rate ranging from about 25-75 ml/minute, about 75-200 ml/minute, about 200-350 ml/minute, about 350-500 ml/minute, about 500-650 ml/minute, about 650-850 ml/minute, or about 850-1000 ml/minute.

30. The process of any one of the preceding claims, wherein the mRNA is mixed in a solution at a flow rate of about 50 ml/minute, about 100 ml/minute, about 150 ml/minute, about 200 ml/minute, about 250 ml/minute, about 300 ml/minute, about 350 ml/minute, about 400 ml/minute, about 450 ml/minute, about 500 ml/minute, about 550 ml/minute, about 600 ml/minute, about 650 ml/minute, about 700 ml/minute, about 750 ml/minute, about 800 ml/minute, about 850 ml/minute, about 900 ml/minute, about 950 ml/minute, or about 1000 ml/minute.

31. The process of any one of the preceding claims, wherein the process comprises a step of first generating an mRNA solution by mixing a citrate buffer with an mRNA stock solution.

32. The process of claim 29, wherein the citrate buffer comprises about 10 mM citrate, about 150 mM NaCl, pH of about 4.5.

33. The process of claim 29 or 30, wherein the mRNA stock solution comprises the mRNA at a concentration at or greater than about 1 mg/ml, about 10 mg/ml, about 50 mg/ml, about 100 mg/ml.

34. The process of any one of claims 29-31, wherein the citrate buffer is mixed at a flow rate ranging between about 100-300 ml/minute, 300-600 ml/minute, 600-1200 ml/minute, 1200-2400 ml/minute, 2400-3600 ml/minute, 3600-4800 ml/minute, or 4800-6000 ml/minute.

35. The process of any one of claims 29-32, wherein the citrate buffer is mixed at a flow rate of about 220 ml/minute, about 600 ml/minute, about 1200 ml/minute, about 2400 ml/minute, about 3600 ml/minute, about 4800 ml/minute, or about 6000 ml/minute.

36. The process of any one of claims 29-33, wherein the mRNA stock solution is mixed at a flow rate ranging between about 10-30 ml/minute, about 30-60 ml/minute, about 60-120 ml/minute, about 120-240 ml/minute, about 240-360 ml/minute, about 360-480 ml/minute, or about 480-600 ml/minute.

37. The process of any one of claims 29-34, wherein the mRNA stock solution is mixed at a flow rate of about 20 ml/minute, about 40 ml/minute, about 60 ml/minute, about 80 ml/minute, about 100 ml/minute, about 200 ml/minute, about 300 ml/minute, about 400 ml/minute, about 500 ml/minute, or about 600 ml/minute.

38. The process of any one of the preceding claims, wherein the lipid nanoparticles encapsulating mRNA are prepared with the pre-formed lipid nanoparticles in a trehalose solution.

39. The process of any one of the preceding claims, wherein the lipid nanoparticles encapsulating mRNA do not require any further downstream processing.

40. A composition of lipid nanoparticles encapsulating mRNA generated by a process of any one of the preceding claims.

41. The process or composition of any one of the preceding claims, wherein the mRNA comprises one or more modified nucleotides.

42. The process or composition of any one of the preceding claims, wherein the mRNA is unmodified.

43. The composition of claim 39, wherein the nanoparticles have a size from about 75 nm to 150 nm.

44. The composition of claim 39, wherein the nanoparticles comprise a PdI from about less than 0.25 to less than 0.16.

45. The composition of claim 39, wherein the dose formulation comprises a concentration of mRNA encapsulated in lipid nanoparticles from about 0.016 mg/kg to 1.0 mg/kg.

46. A method of delivering mRNA for *in vivo* protein production comprising administering into a subject a composition of lipid nanoparticles encapsulating mRNA generated by a process of any one of claims 1-39, wherein the mRNA encodes a protein of interest.

47. A method of delivering mRNA for *in vivo* protein production comprising

administering into a subject the composition of any one of claims 40-45.

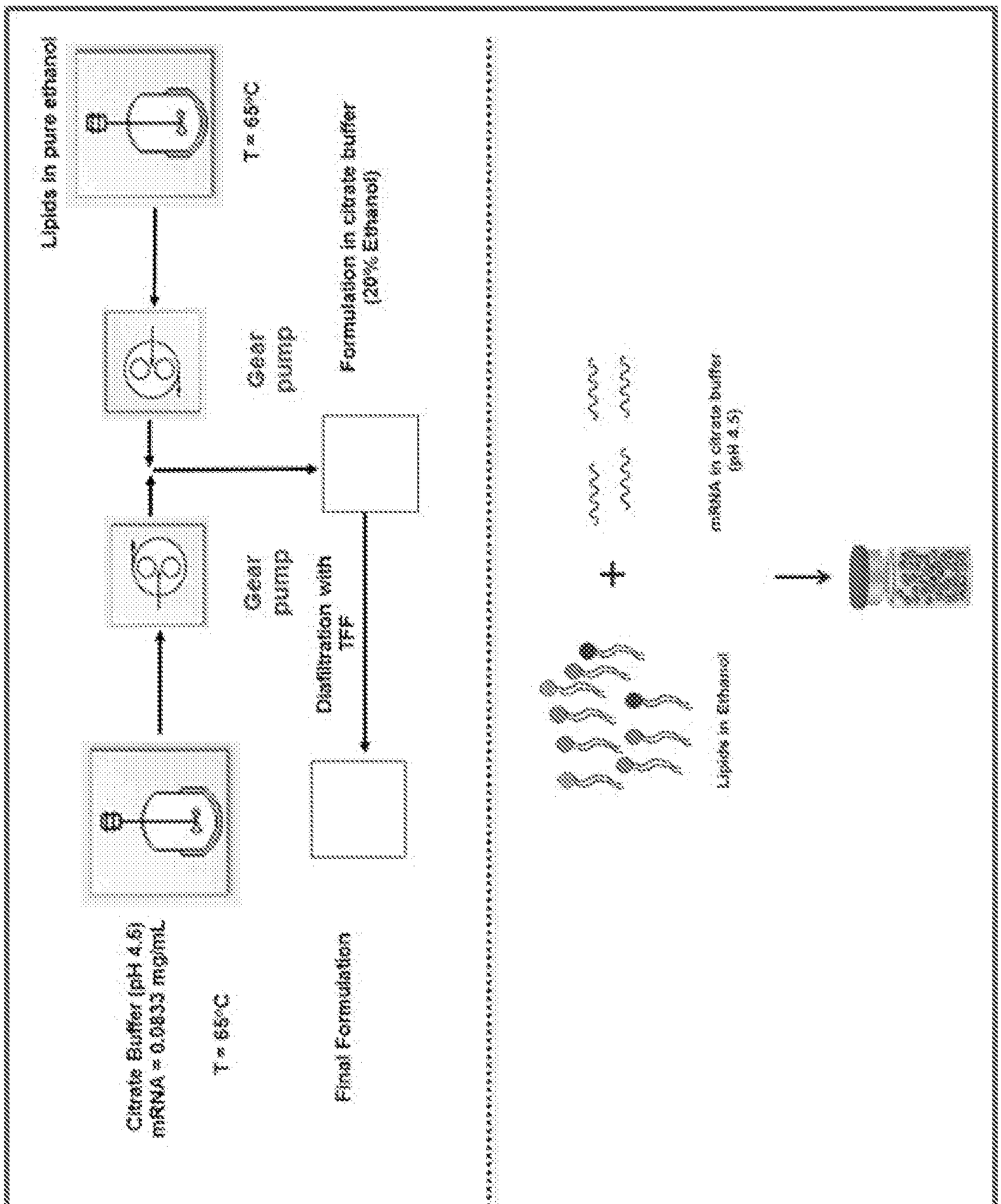

Lipids in pure ethanol
T = 65°C
Gear pump
Formulation in citrate buffer (20% Ethanol)
Gear pump
Diafiltration with TFF
Final Formulation
Citrate Buffer (pH 4.0) mRNA = 0.0833 mg/mL
T = 65°C
mRNA in citrate buffer (pH 4.5)
Lipids in Ethanol

FIG. 1

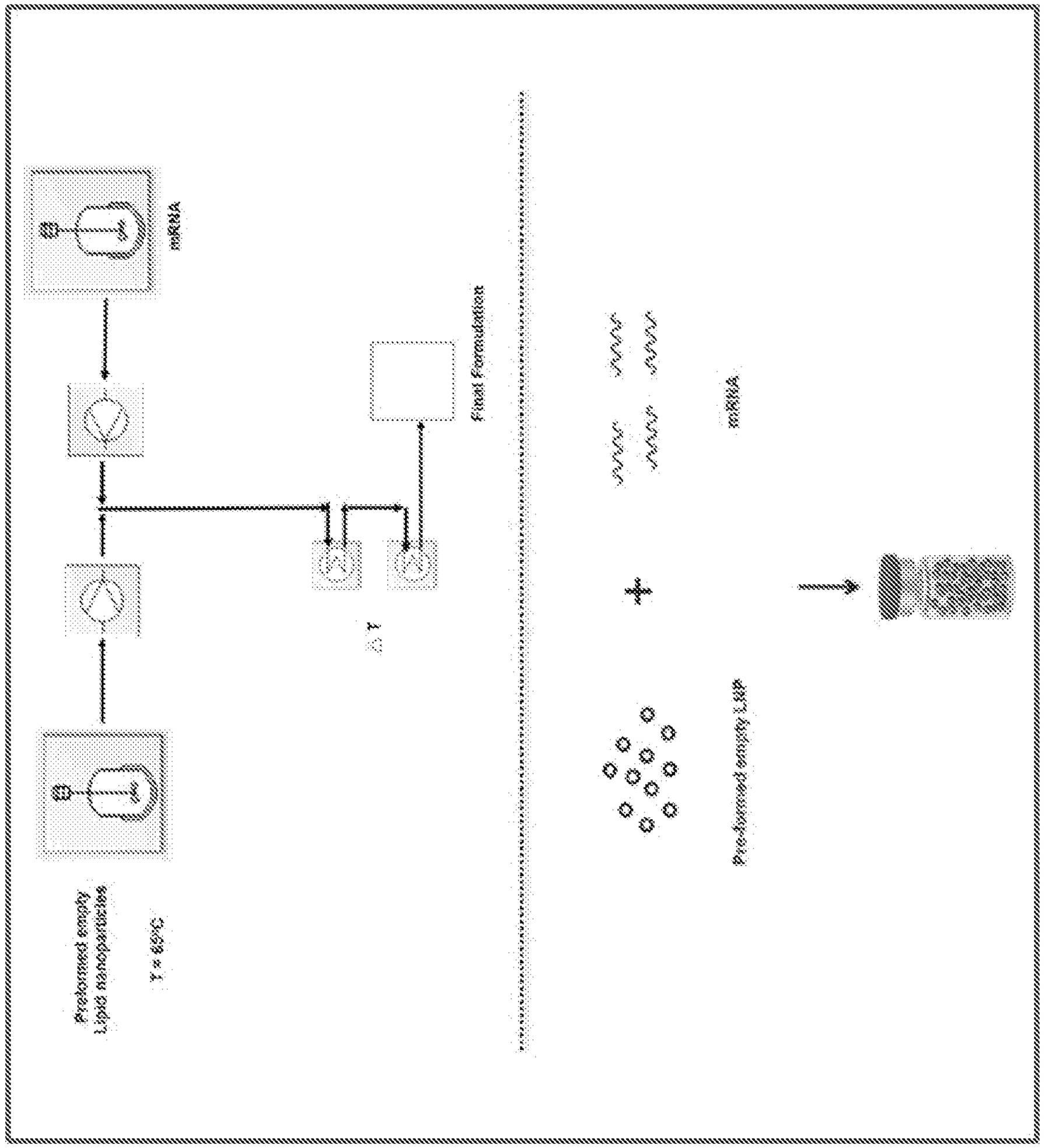

mRNA
Final Formulation
mRNA
Pre-formed empty LNP
Pre-formed empty LNP
mRNA
Pre-formed empty LNP
Pre-formed empty LNP
mRNA

FIG. 2

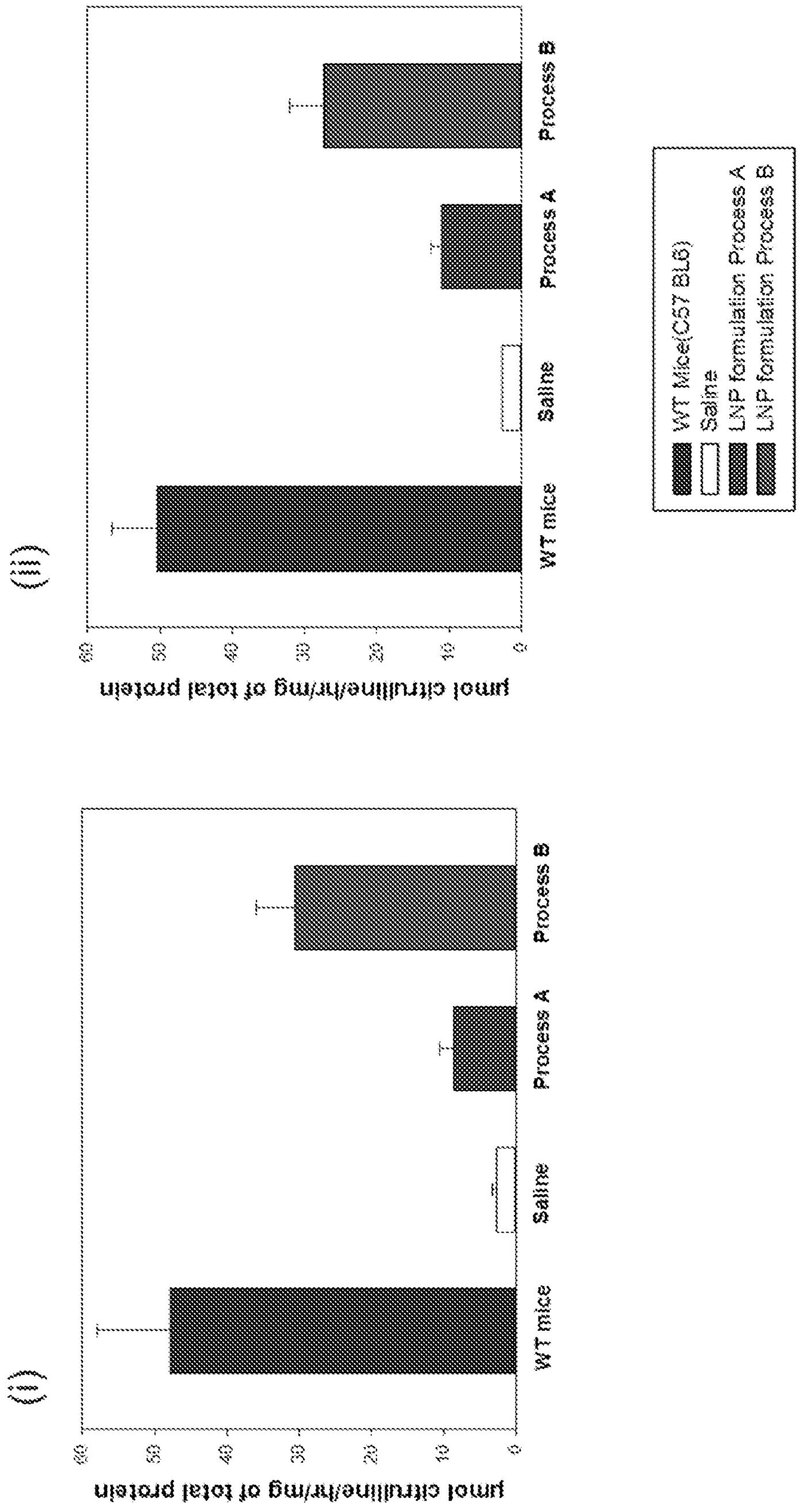

(iii)
μmol citrulline/hr/mg of total protein
0
10
20
30
40
50
60
WT mice
Saline
Process A
Process B
WT Mice(C57 BL6)
Saline
LNP formulation Process A
LNP formulation Process B

FIG. 3

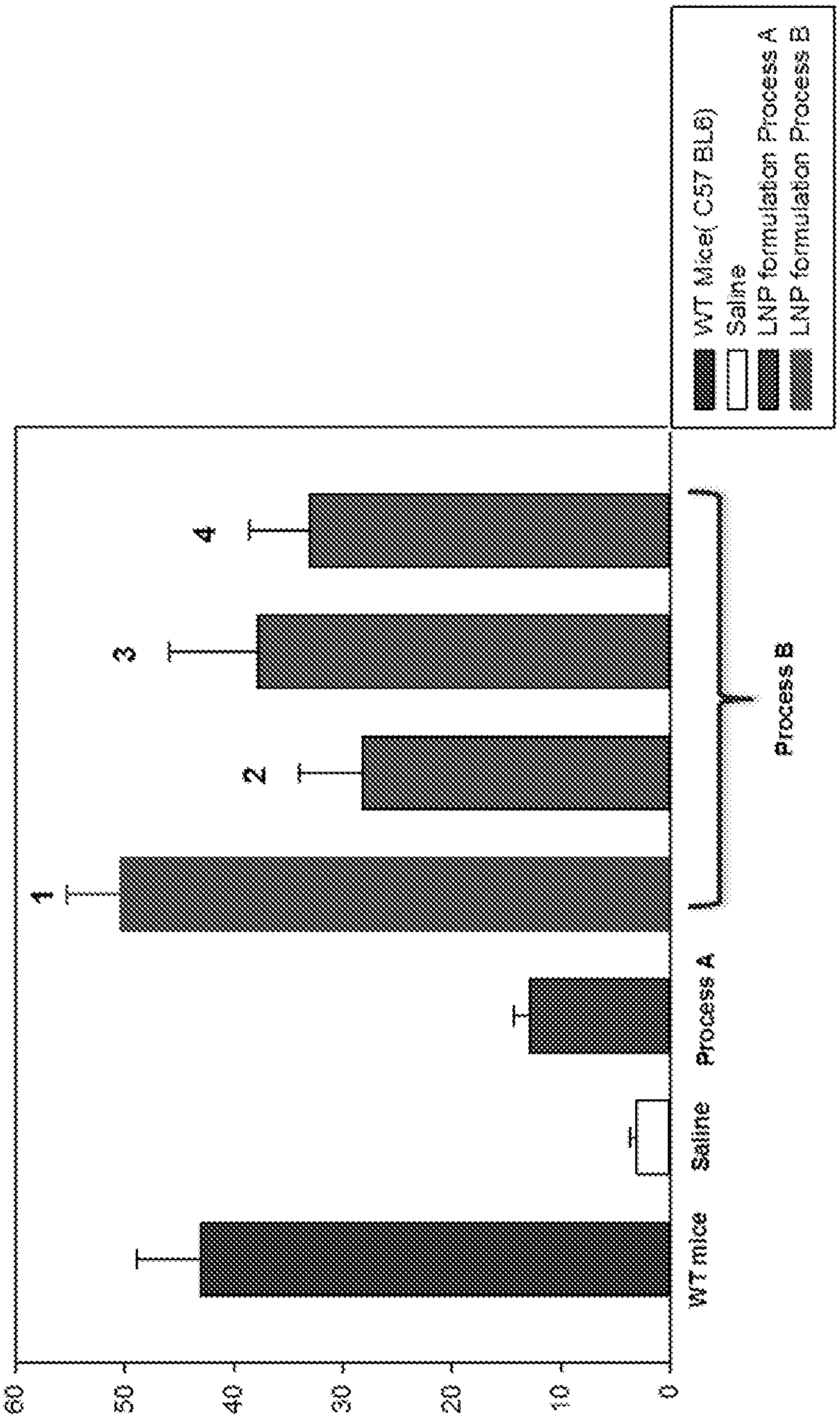

WT Mice(C57 BL6)
Saline
LNP formulation Process A
LNP formulation Process B
Process B
Process A
Saline
WT mice
1
2
3
4
60
50
40
30
20
10
0
µmol citrulline/hr/mg of total protein

FIG. 4

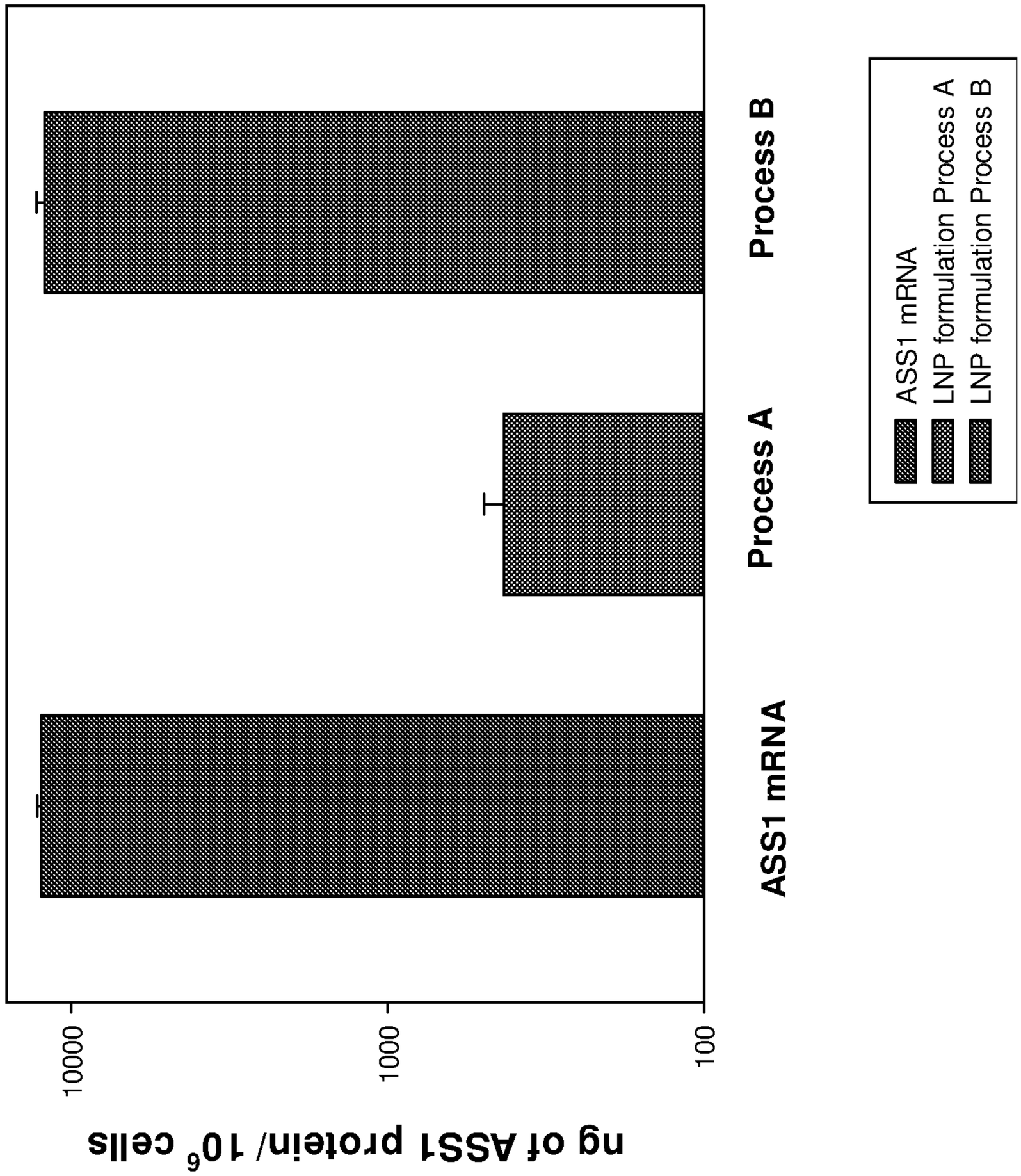
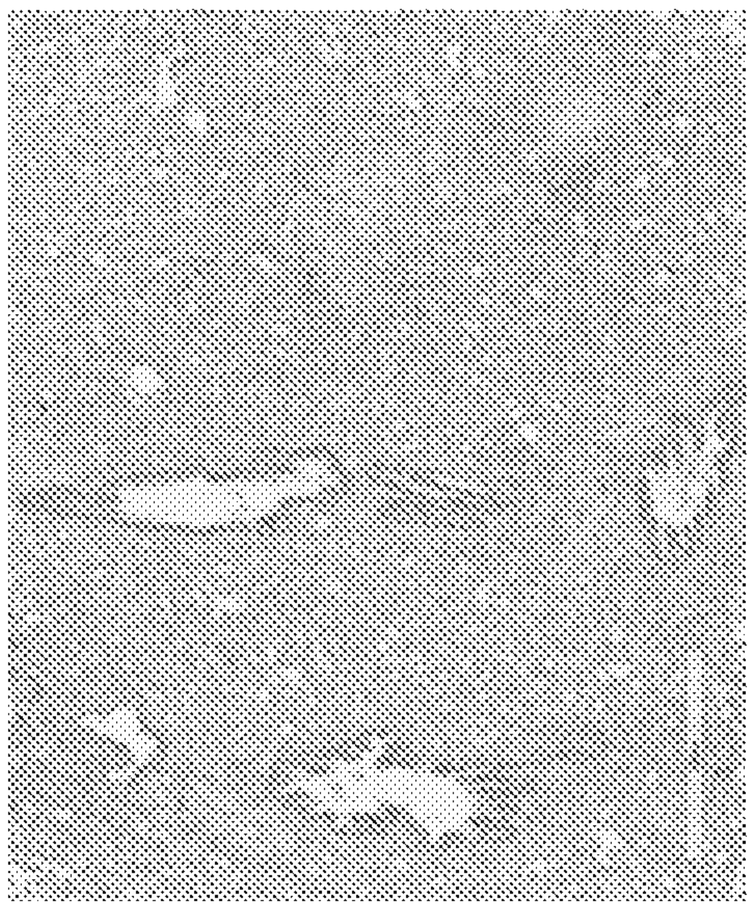
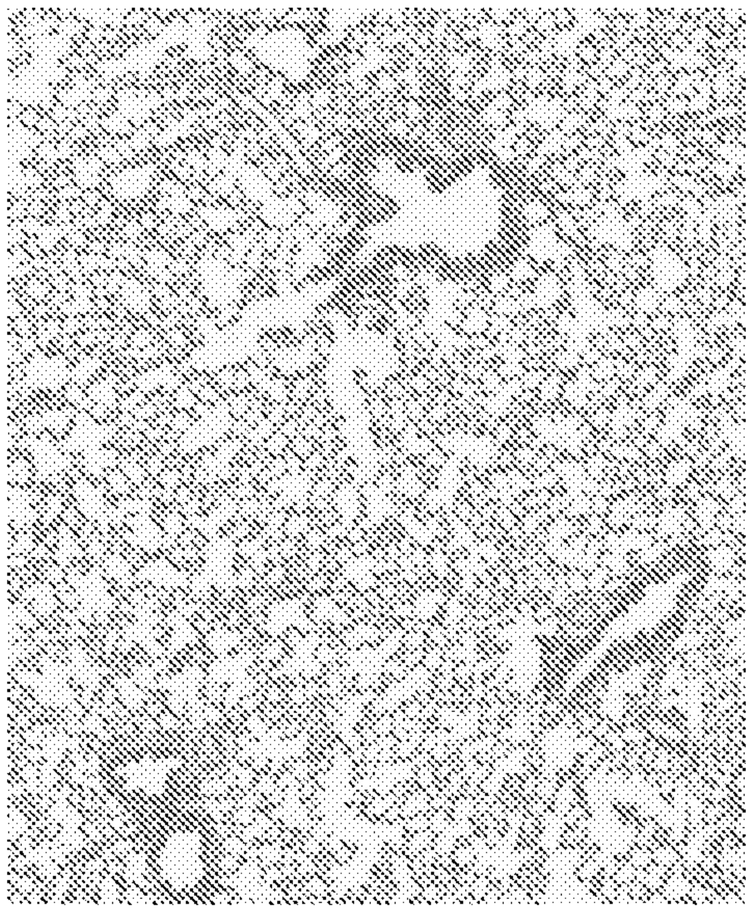

ng of ASS1 protein/ 10^6 cells
10000
1000
100
ASS1 mRNA
Process A
Process B
ASS1 mRNA
LNP formulation Process A
LNP formulation Process B

FIG. 5







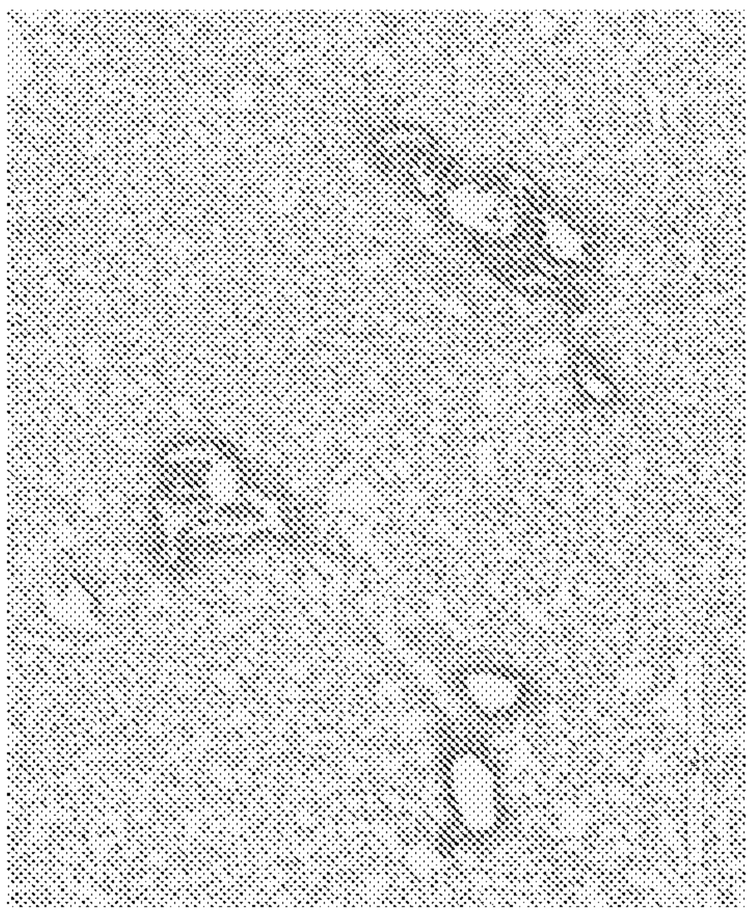


FIG. 6

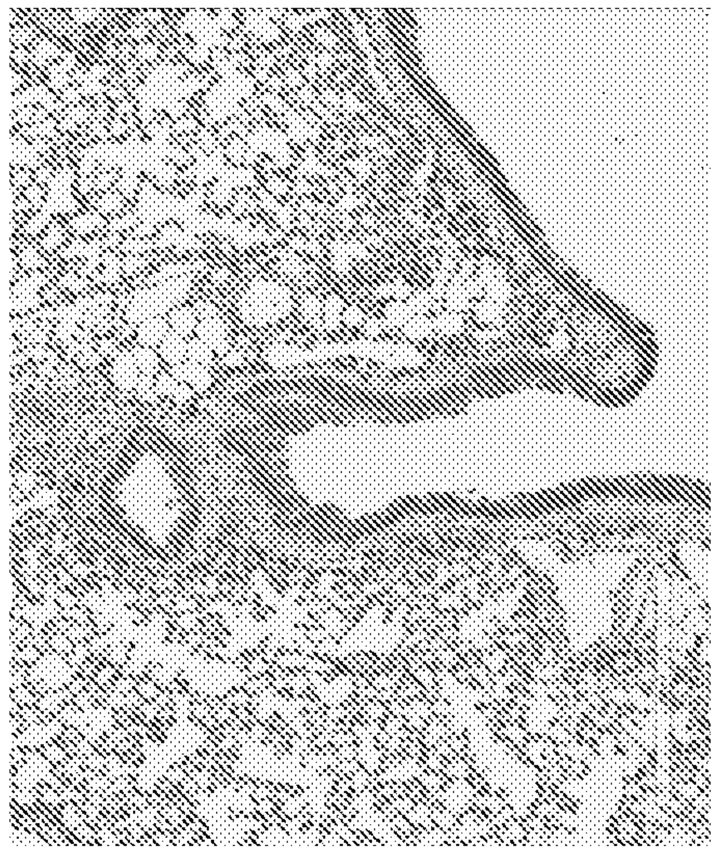


FIG. 7


Color Scale
Min = 1.12e5
Max = 3.98e5
Pressure
(pascal/cm^2)
3.5
3.0
2.5
2.0
1.5
x10^6

FIG. 8

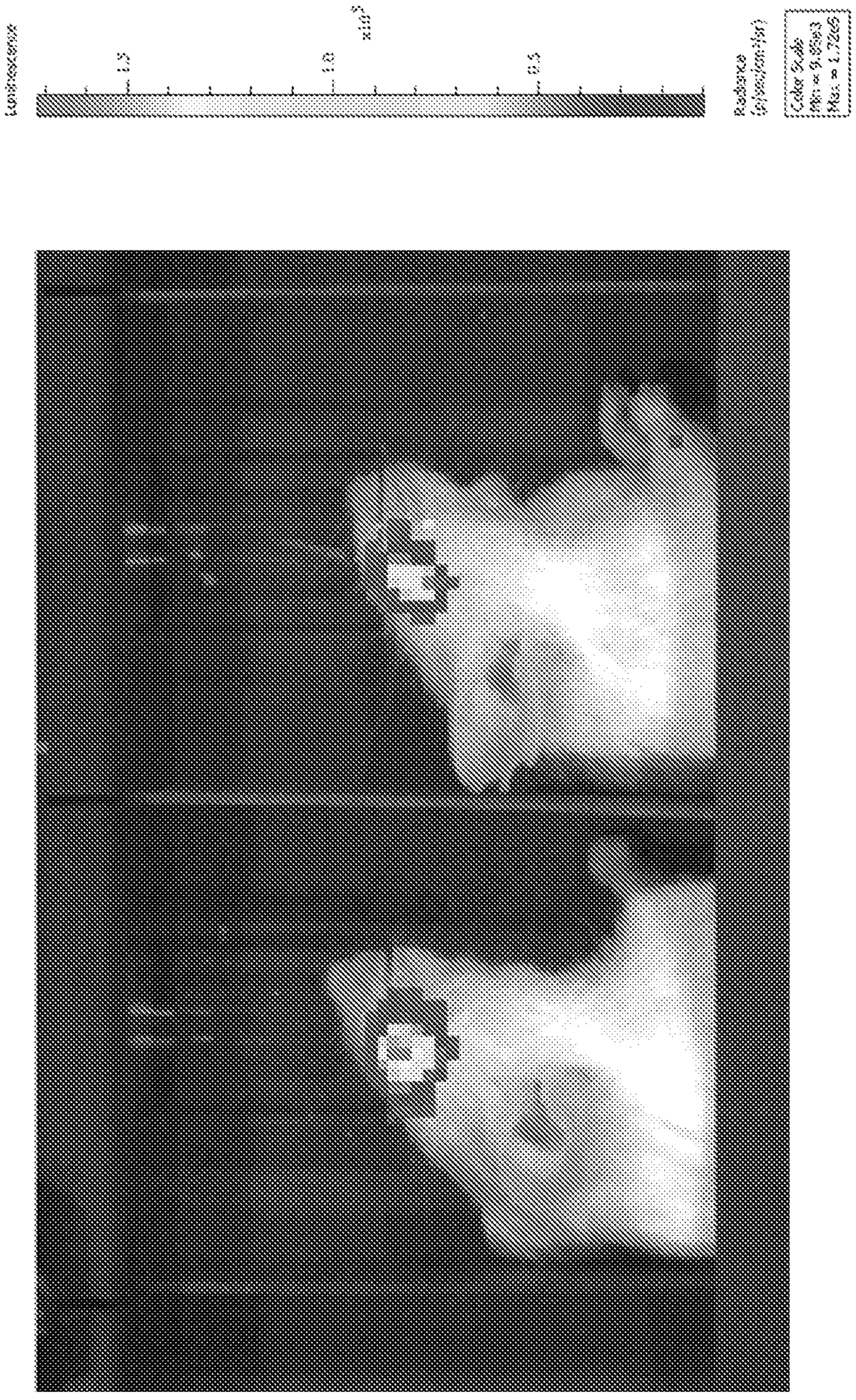

Luminescence
1.5
1.0
0.5
x10^5
Radiance
(p/s/sr/cm^2/cm^-1)
Color Scale
Min = 9.85e3
Max = 1.72e5

FIG. 9

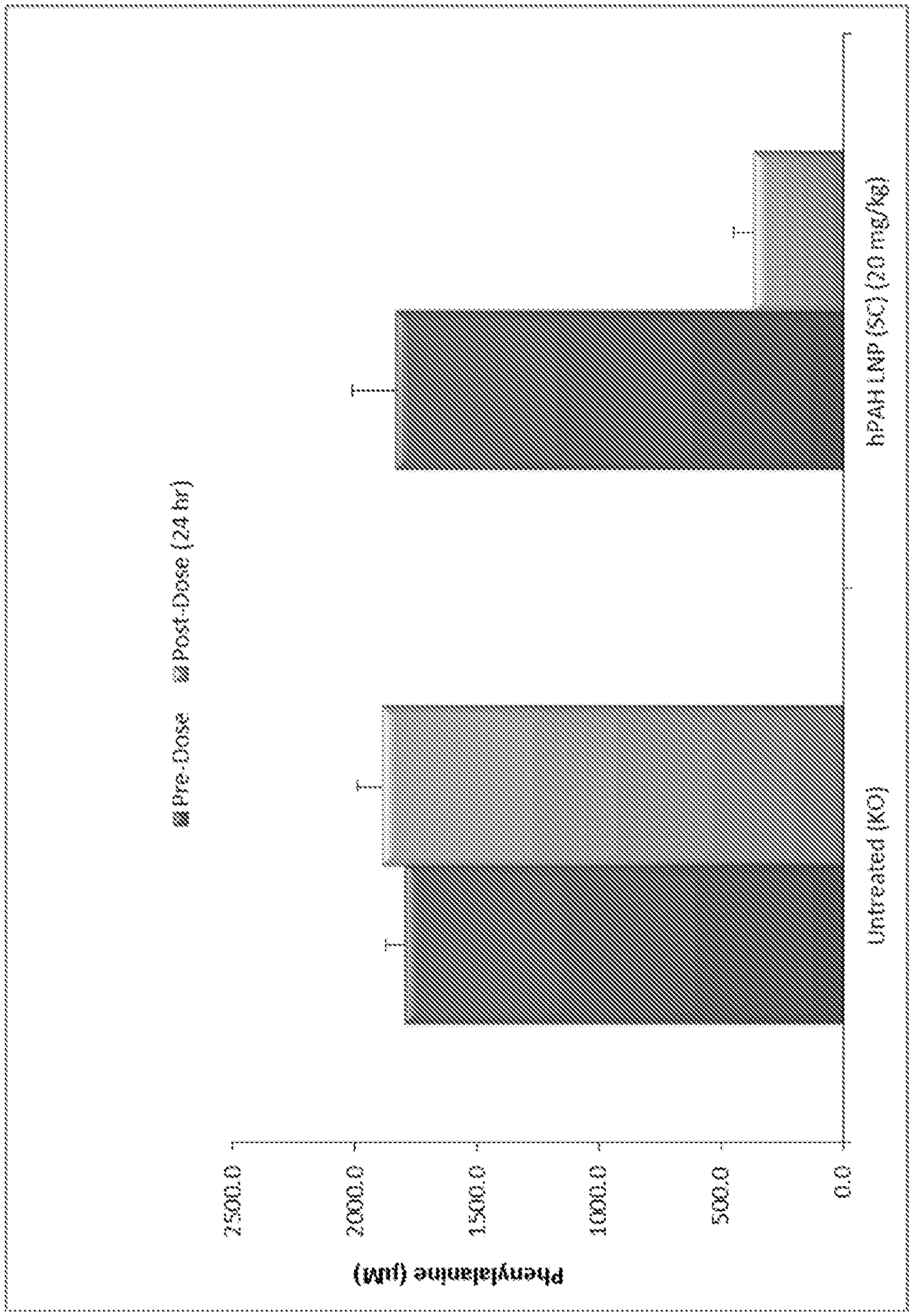

Phenylalanine (μM)
2500.0
2000.0
1500.0
1000.0
500.0
0.0
Untreated (KO)
Pre-Dose
Post-Dose (24 hr)
hPAH NP (SC) (20 mg/kg)

FIG. 10

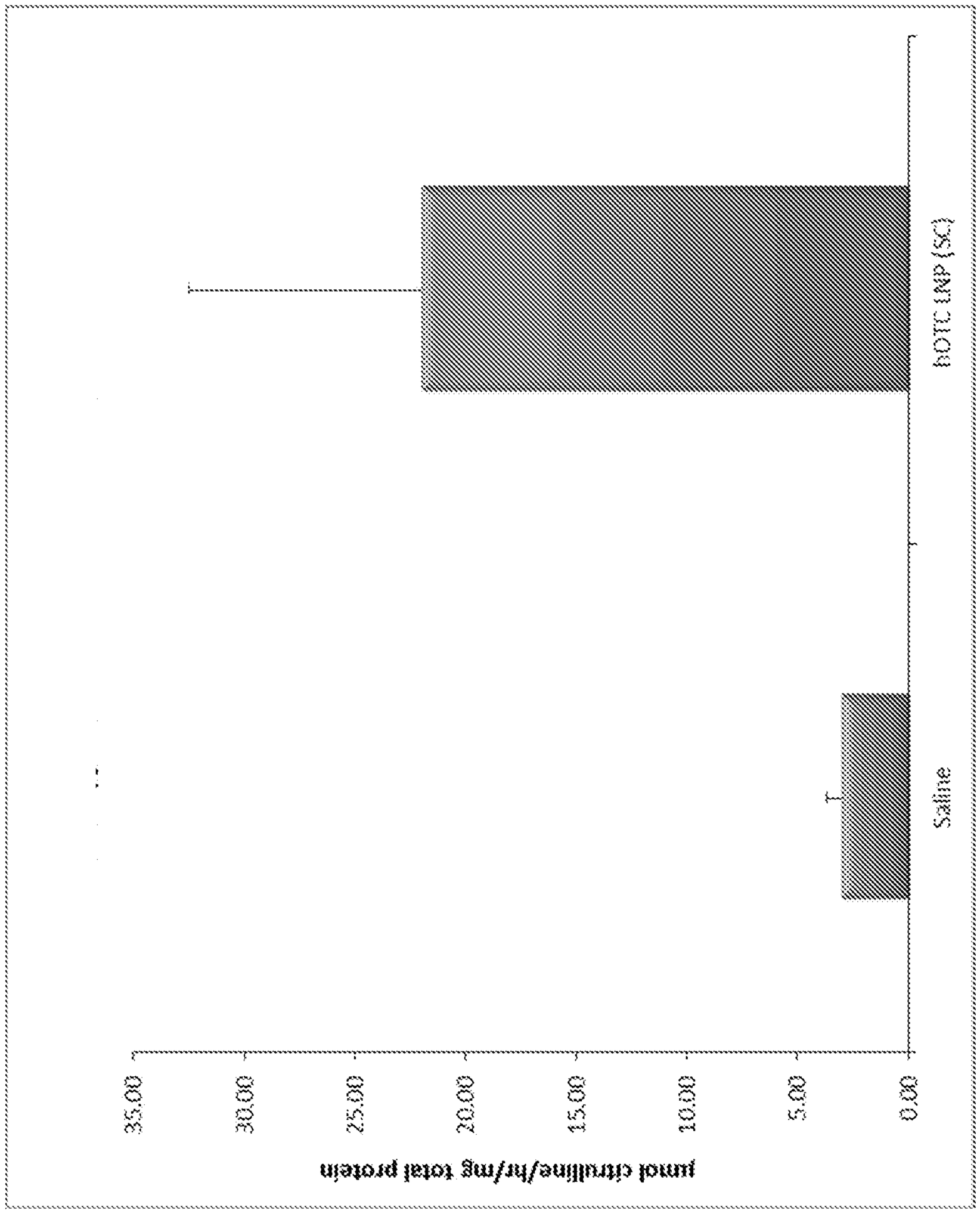

bOTC IMP (SC)
Saline
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
µmol citrulline/hr/mg total protein

FIG. 11

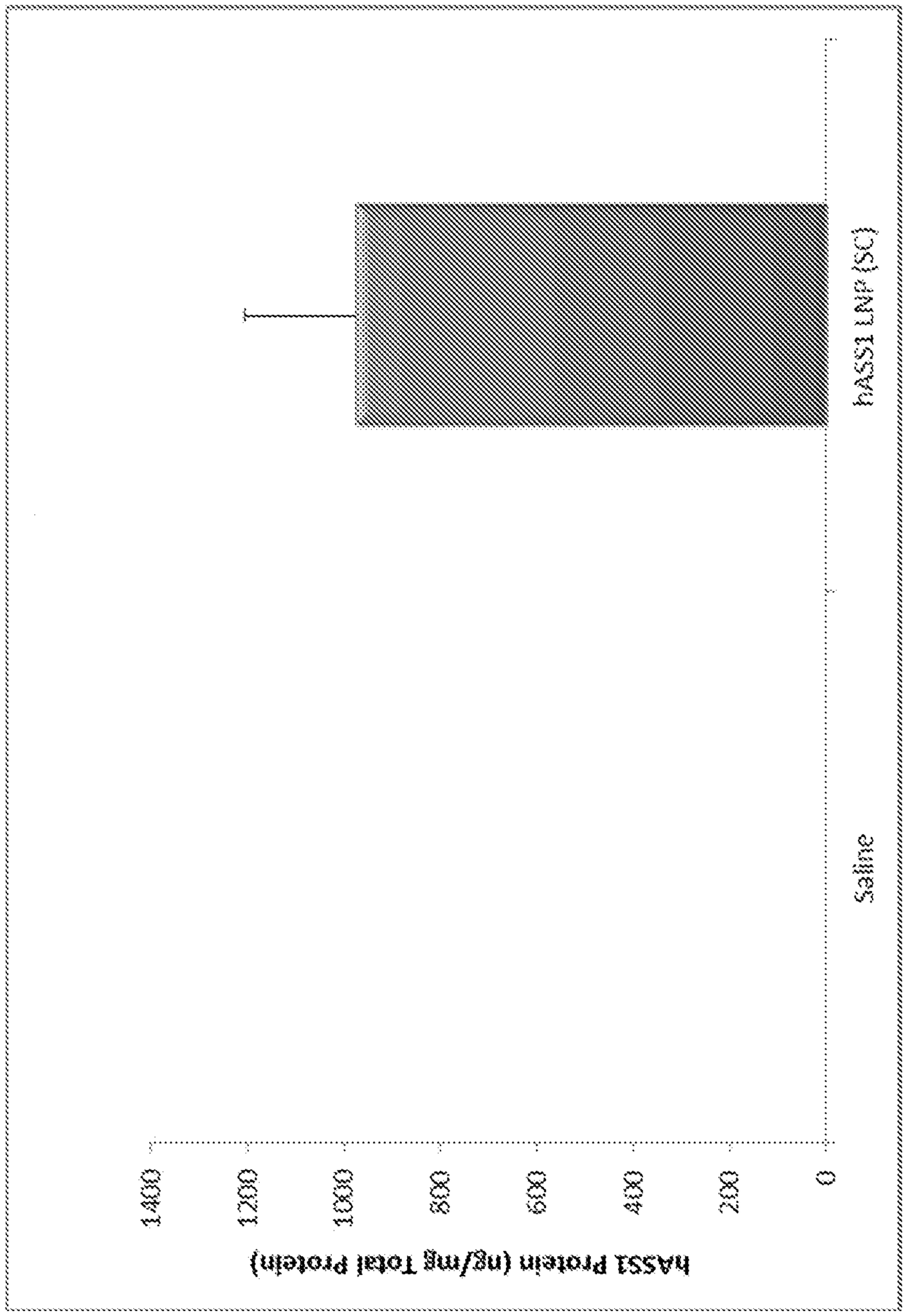

hASS1 LNP (SC)
Saline
hASS1 Protein (ng/mg Total Protein)
0
200
400
600
800
1000
1200
1400

FIG. 12

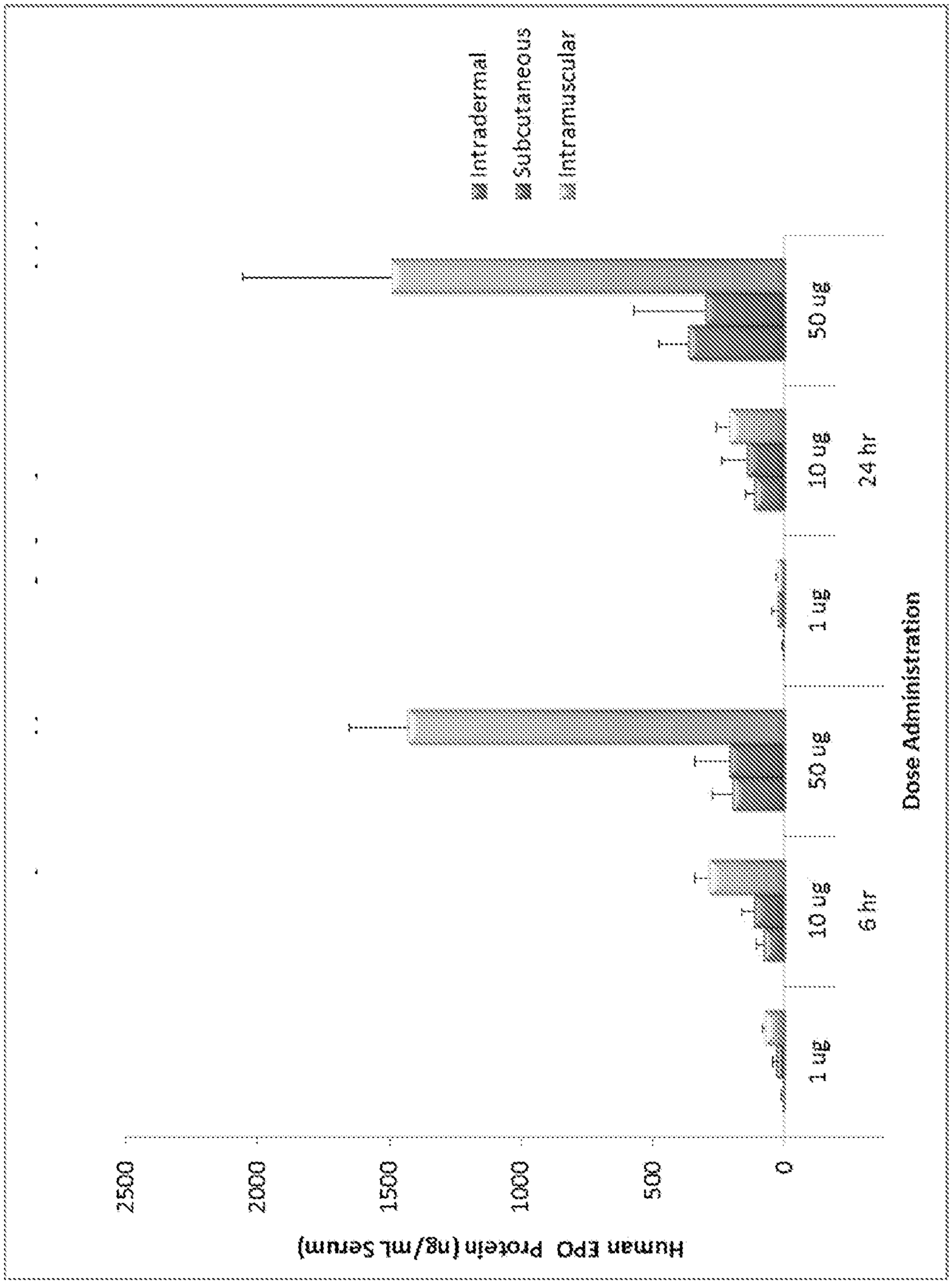

Intradermal
Subcutaneous
Intramuscular
1 ug
10 ug
50 ug
24 hr
1 ug
10 ug
50 ug
6 hr
Dose Administration
2500
2000
1500
1000
500
0
Human EPO Protein (ng/mL Serum)

FIG. 13

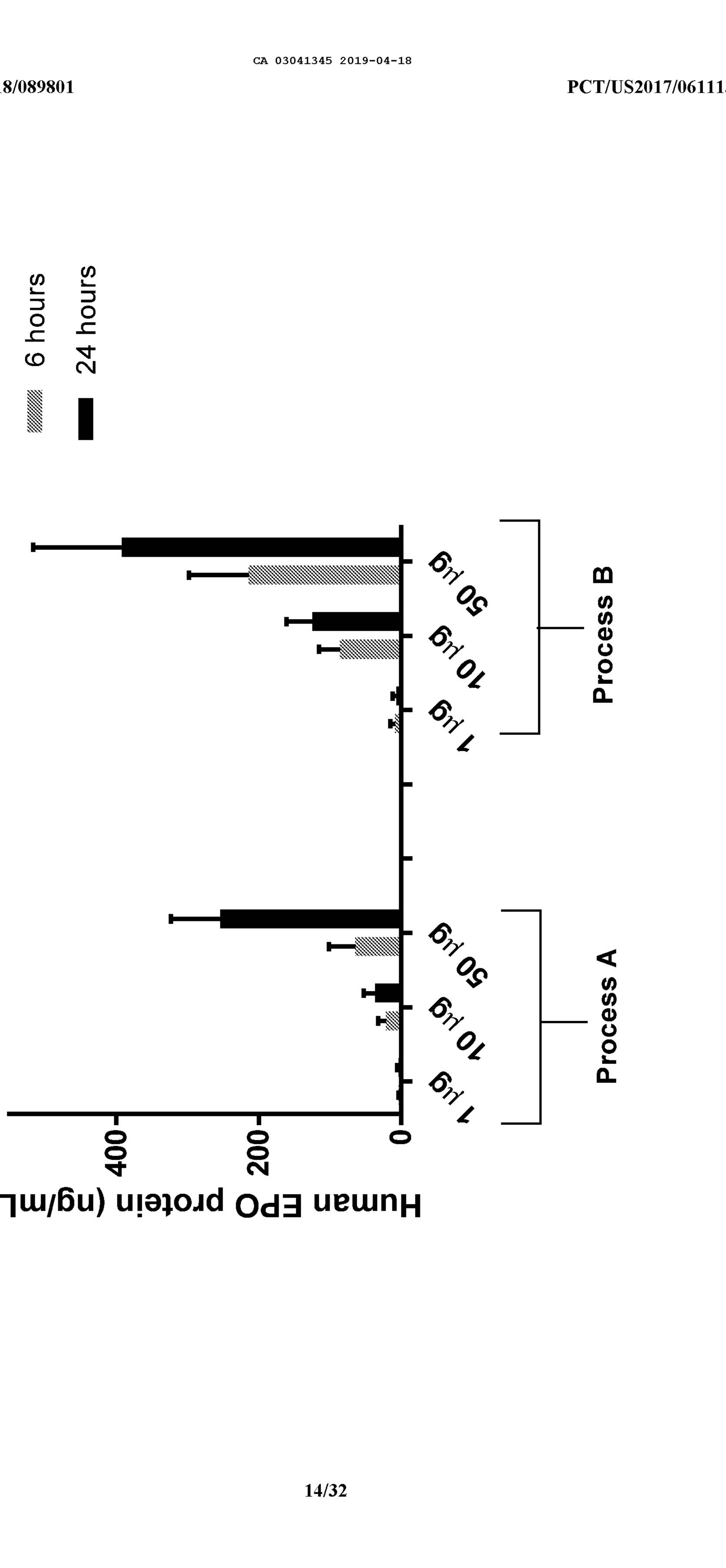

6 hours
24 hours
50 µg
10 µg
1 µg
Process B
50 µg
10 µg
1 µg
Process A
0
200
400
Human EPO protein (ng/mL)

FIG. 14

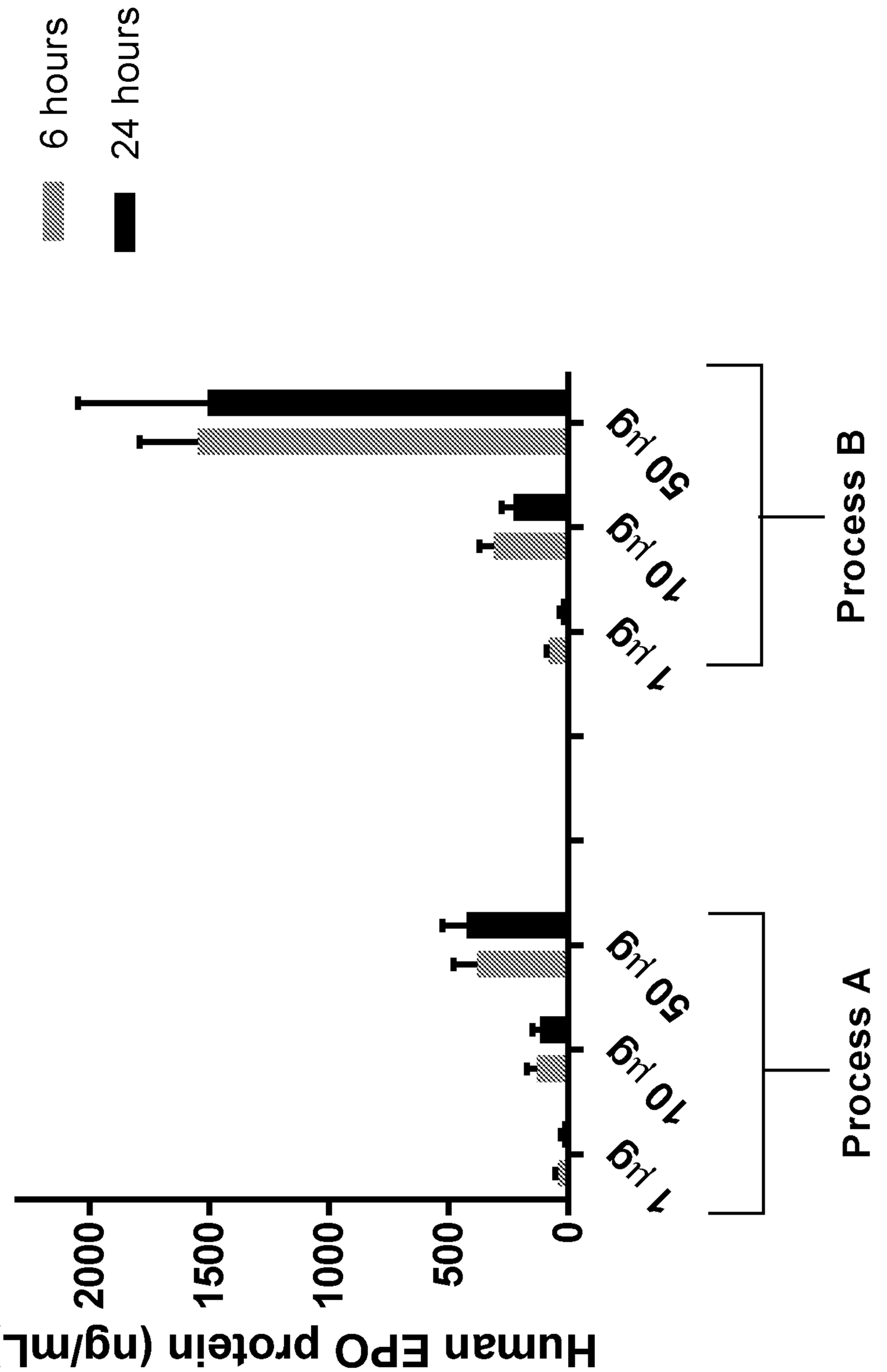

6 hours
24 hours
Process B
Process A
1 µg
10 µg
50 µg
1 µg
10 µg
50 µg
0
500
1000
1500
2000
Human EPO protein (ng/mL)

FIG. 15

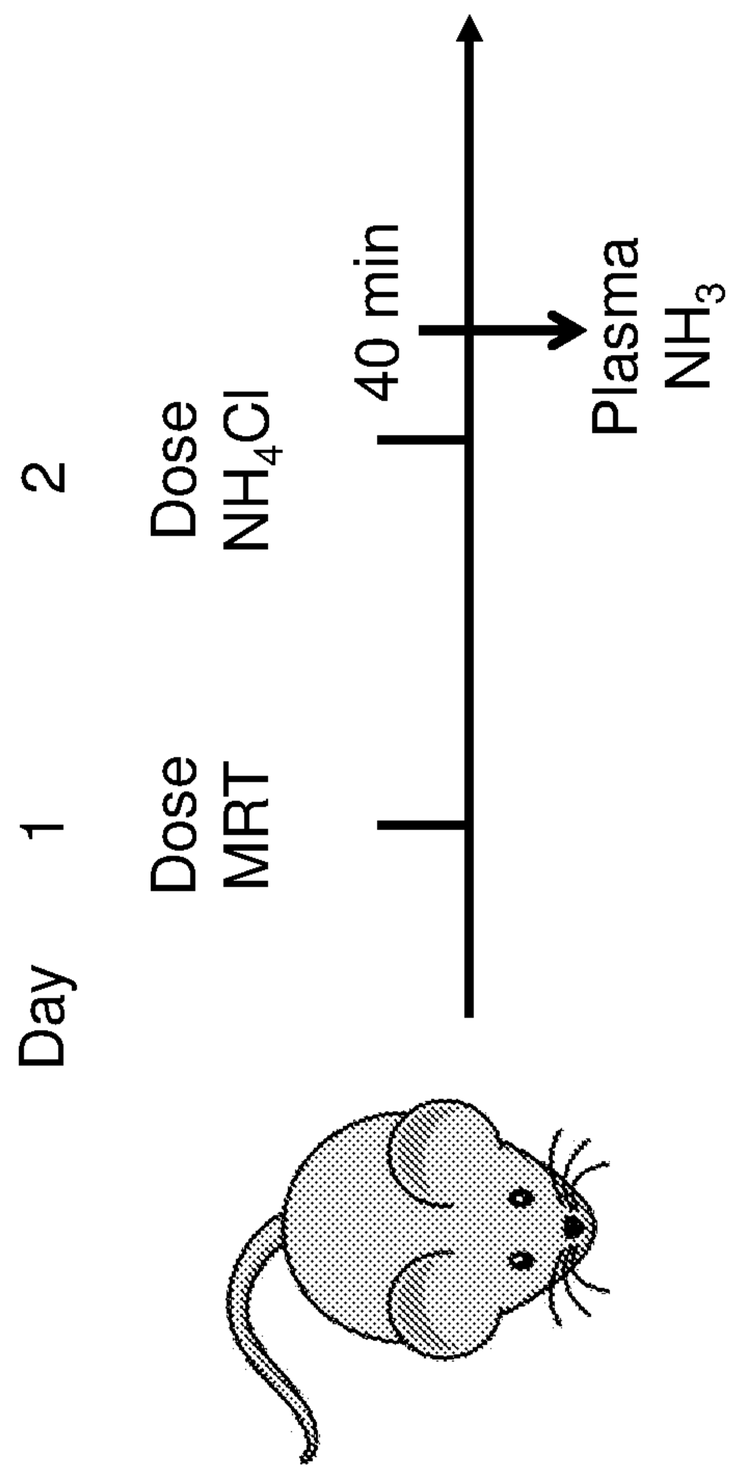


FIG. 16

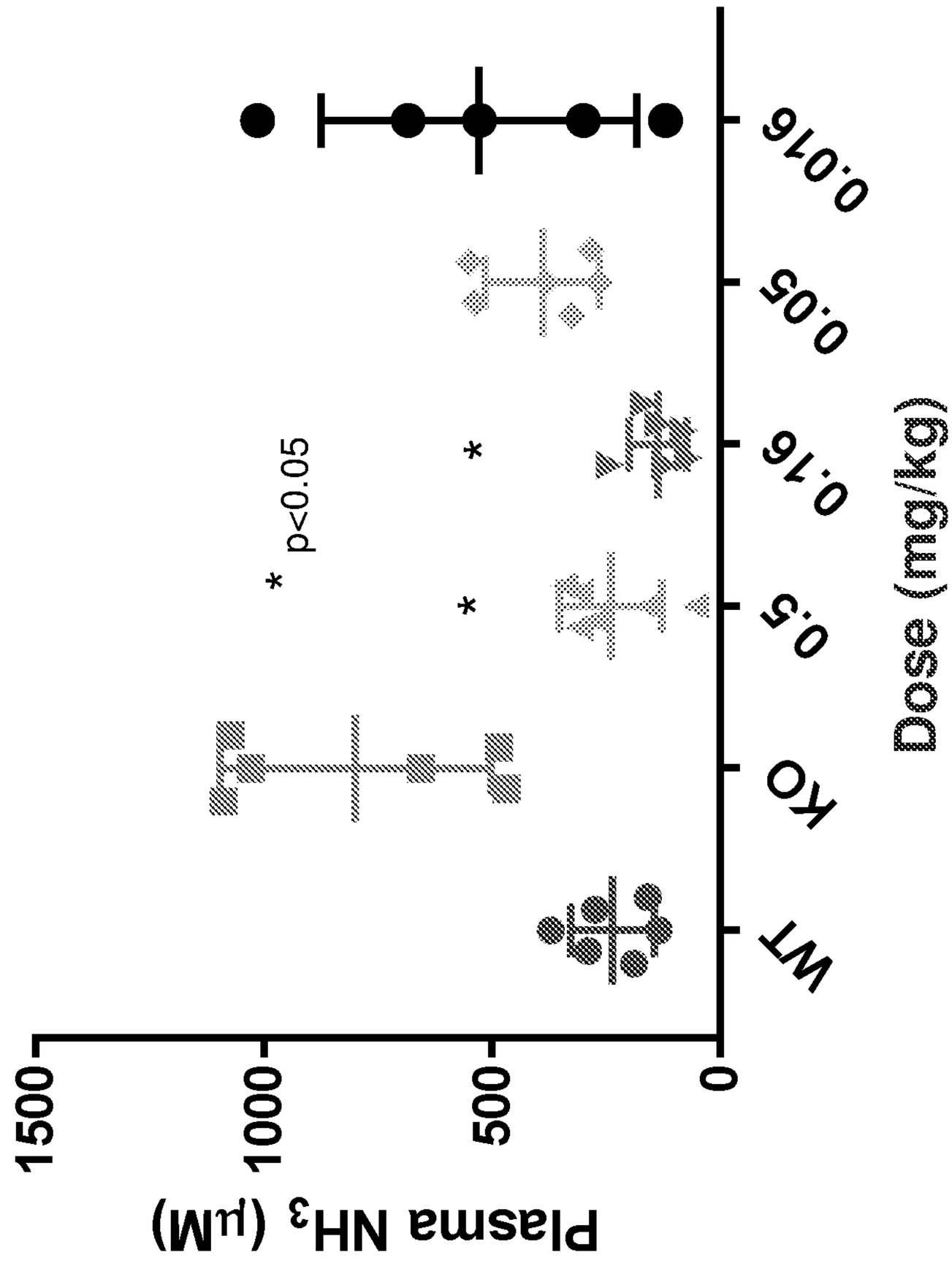

Plasma NH3 (μM)
1500
1000
500
0
WT
KO
0.5
0.16
0.05
0.016
Dose (mg/kg)
* p<0.05
*
*

FIG. 17

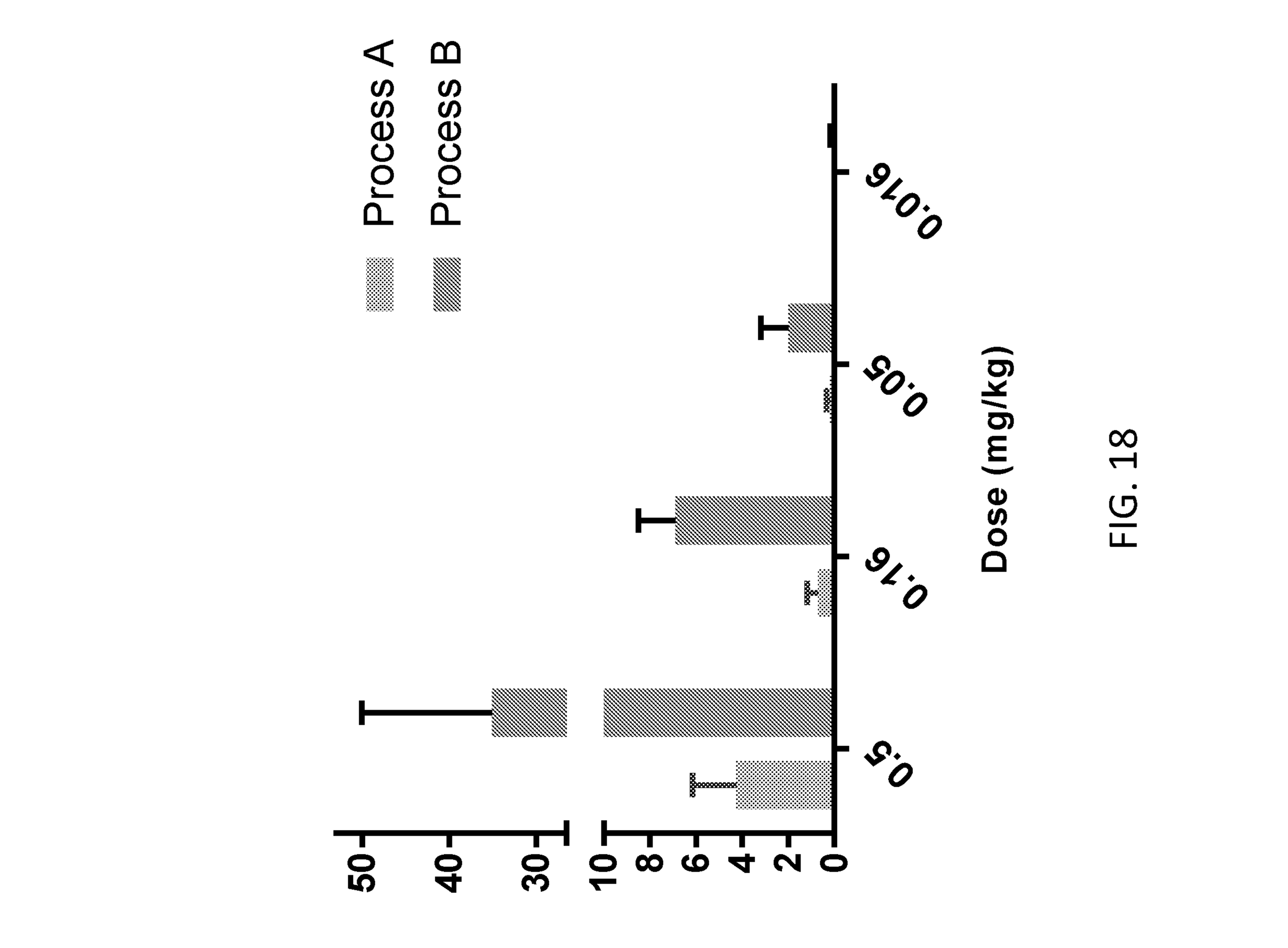

Process A
Process B
0.016
0.05
0.16
0.5
50
40
30
10
8
6
4
2
0
Dose (mg/kg)
µg hOTC/mg Protein

FIG. 18

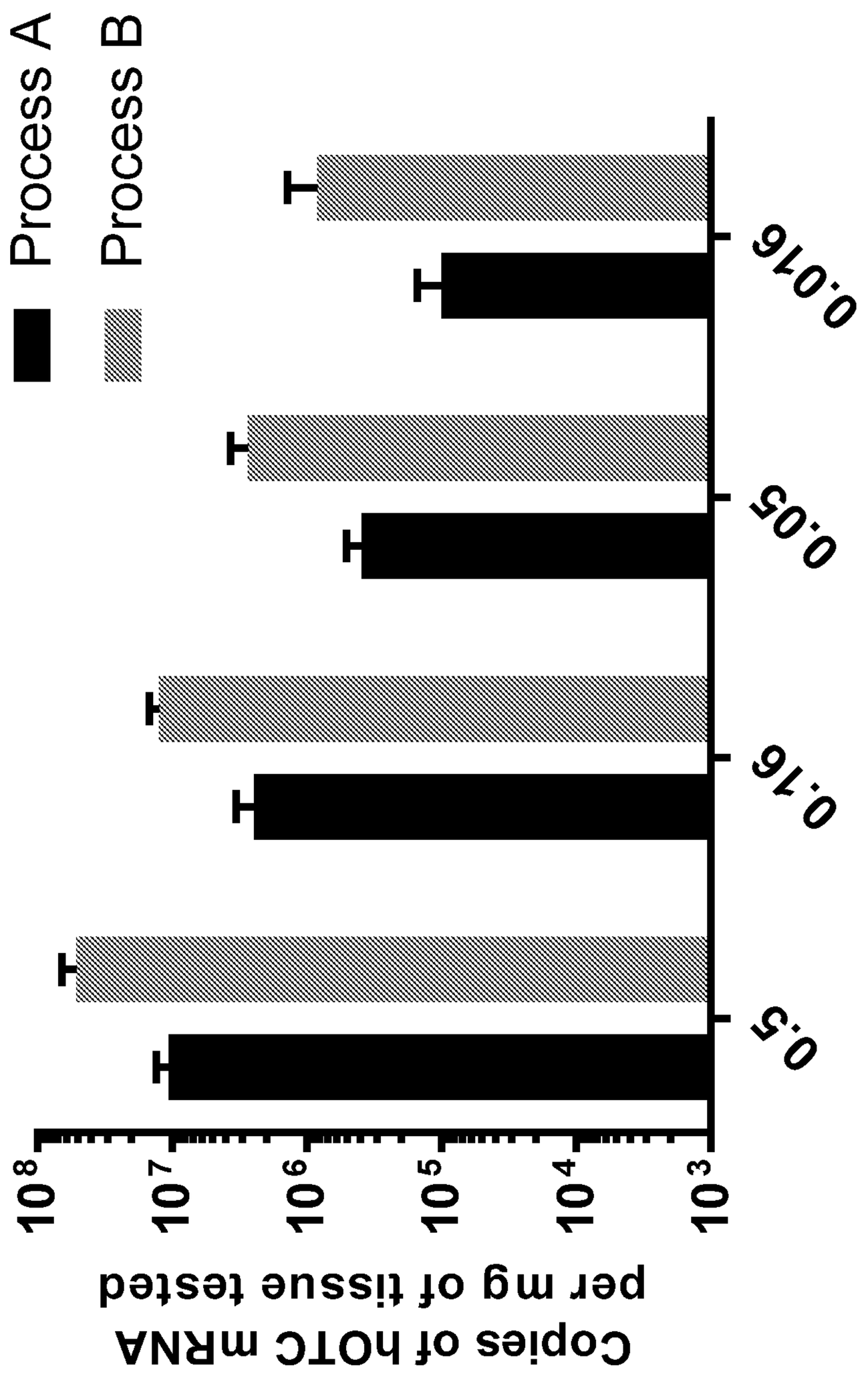

Process A
Process B
Copies of hOTC mRNA per mg of tissue tested
10^8
10^7
10^6
10^5
10^4
10^3
0.5
0.16
0.05
0.016
Dose (mg/kg)

FIG. 19

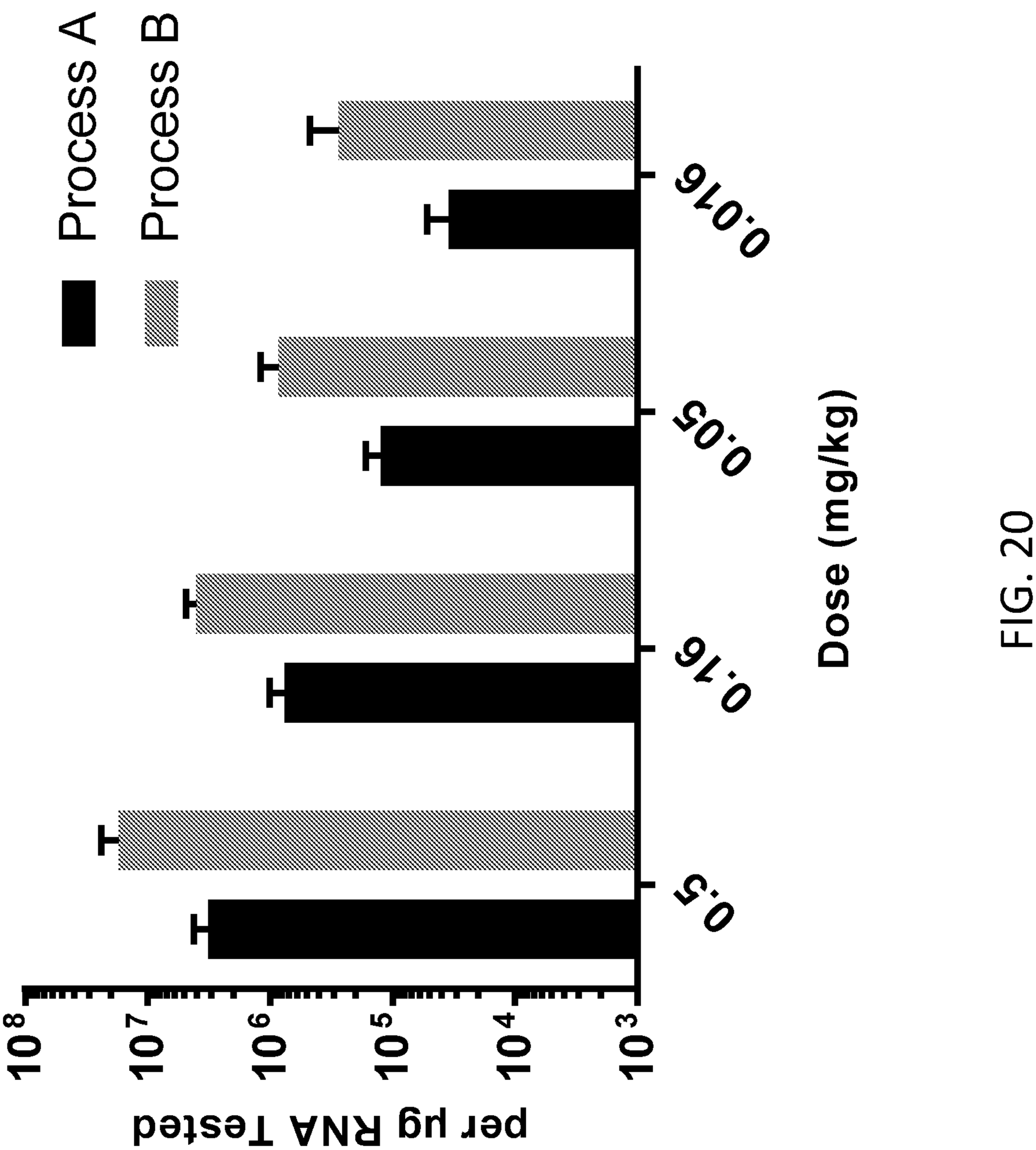

A
B
Process
Process
0.016
0.05
0.16
0.5
Dose (mg/kg)
10^8
10^7
10^6
10^5
10^4
10^3
per µg RNA Tested
Copies of hOTC mRNA

FIG. 20

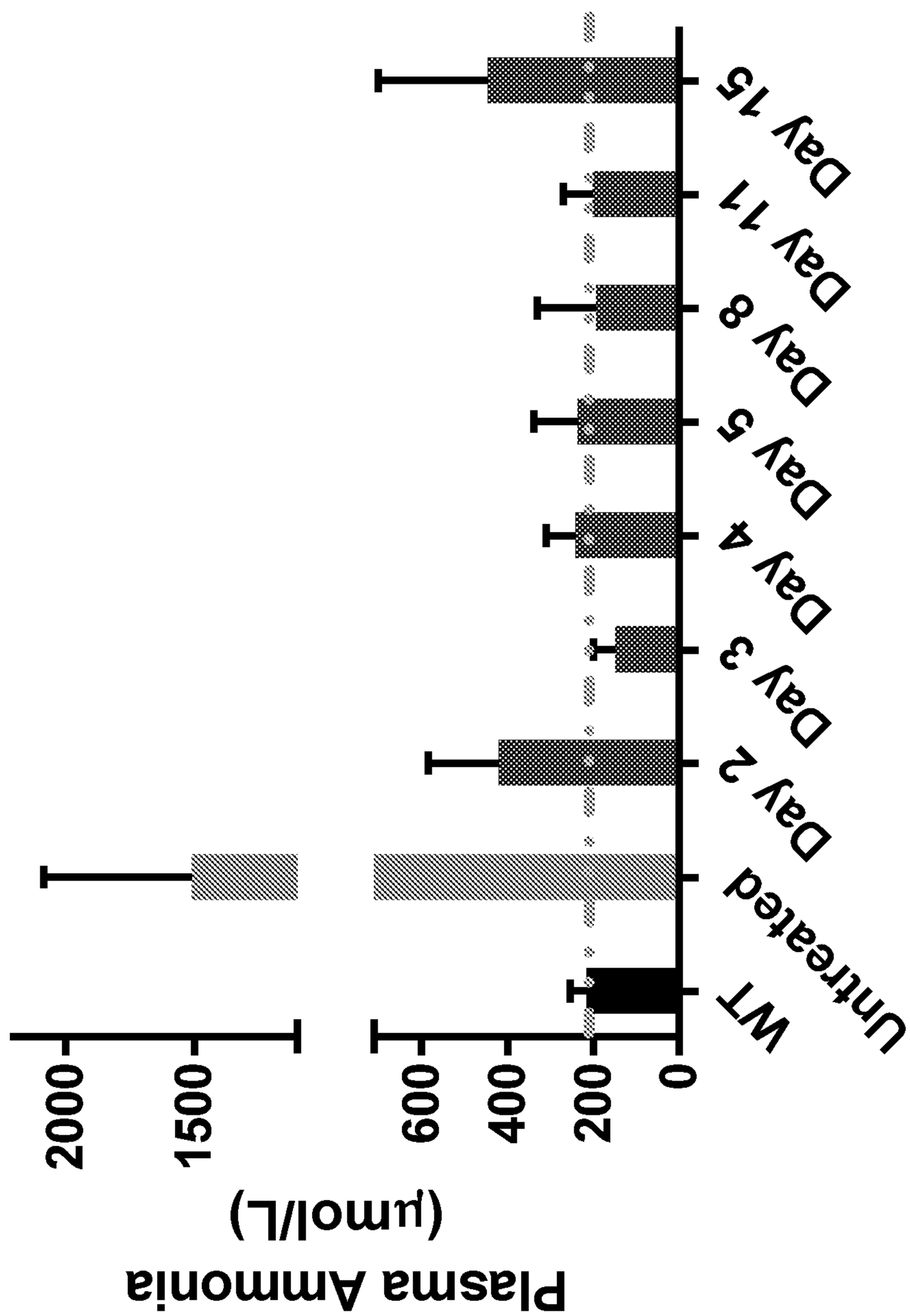

Plasma Ammonia
(μmol/L)
2000
1500
600
400
200
0
WT
Untreated
Day 2
Day 3
Day 4
Day 5
Day 8
Day 11
Day 15
Time

FIG. 21

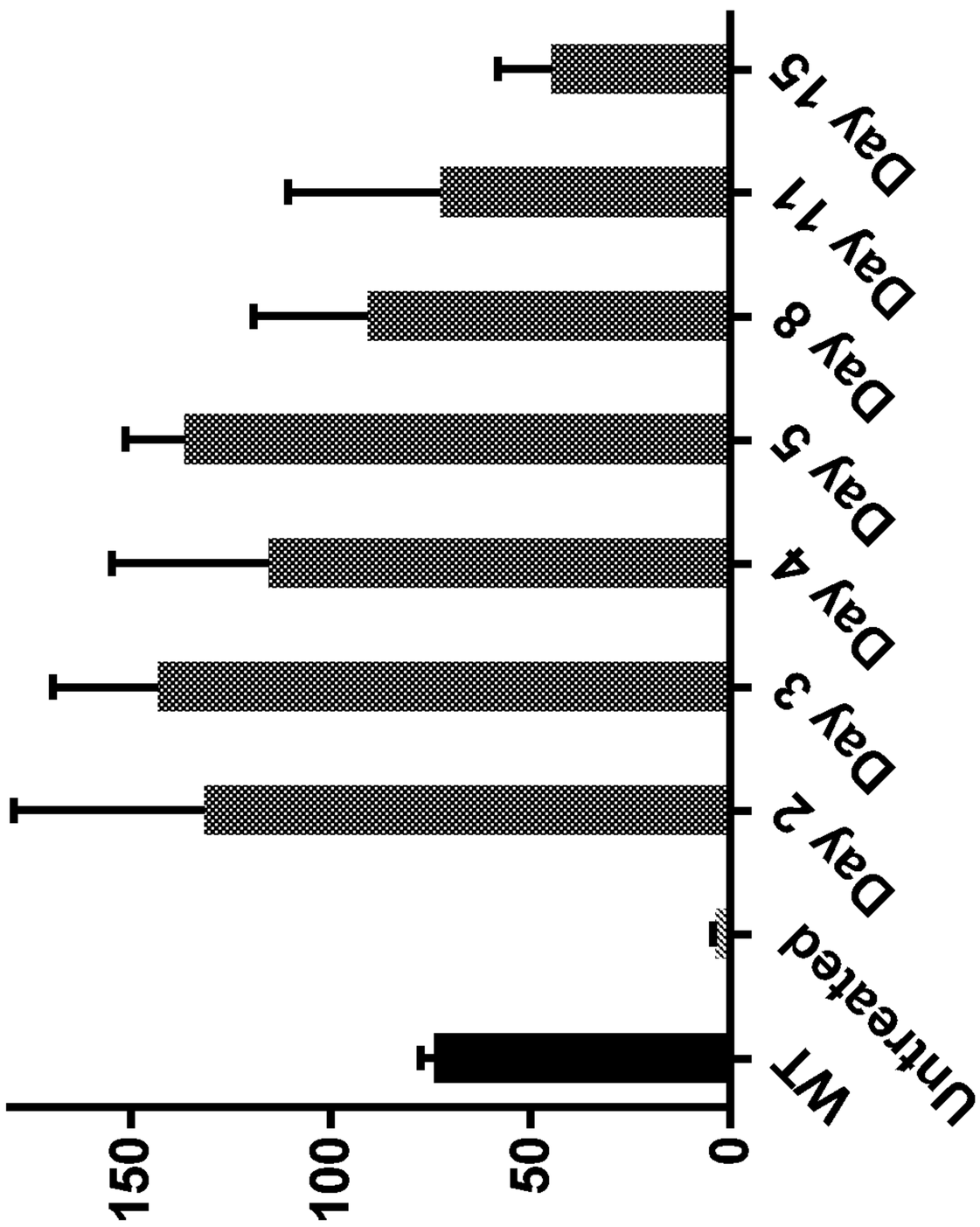

μmol citrulline /hr/mg
of total protein
150
100
50
0
WT
Untreated
Day 2
Day 3
Day 4
Day 5
Day 8
Day 11
Day 15
Time

FIG. 22

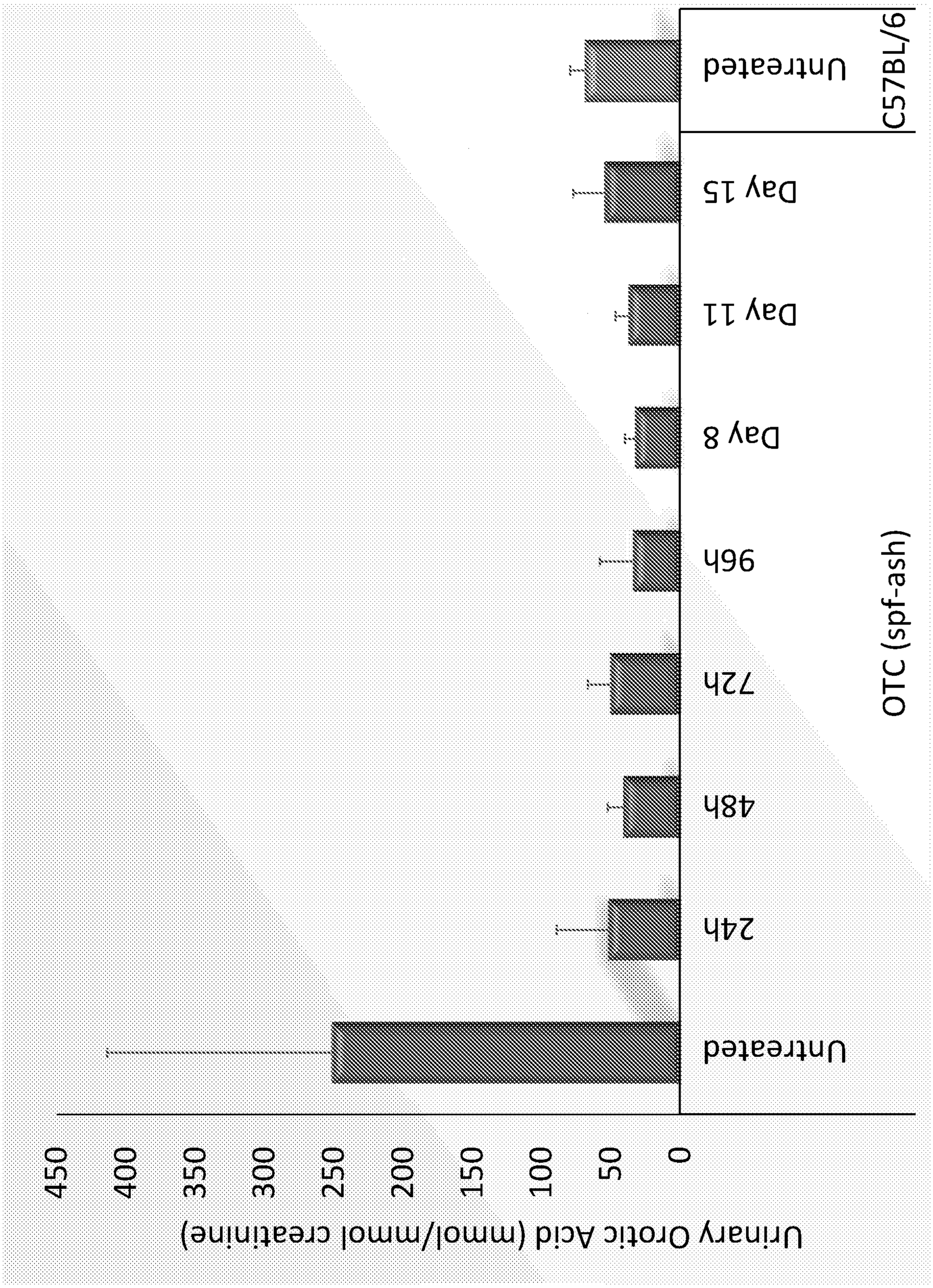

C57BL/6
Untreated
Day 15
Day 11
Day 8
96h
72h
48h
24h
Untreated
OTC (spf-ash)
Urinary Orotic Acid (mmol/mmol creatinine)
450
400
350
300
250
200
150
100
50
0

FIG. 23

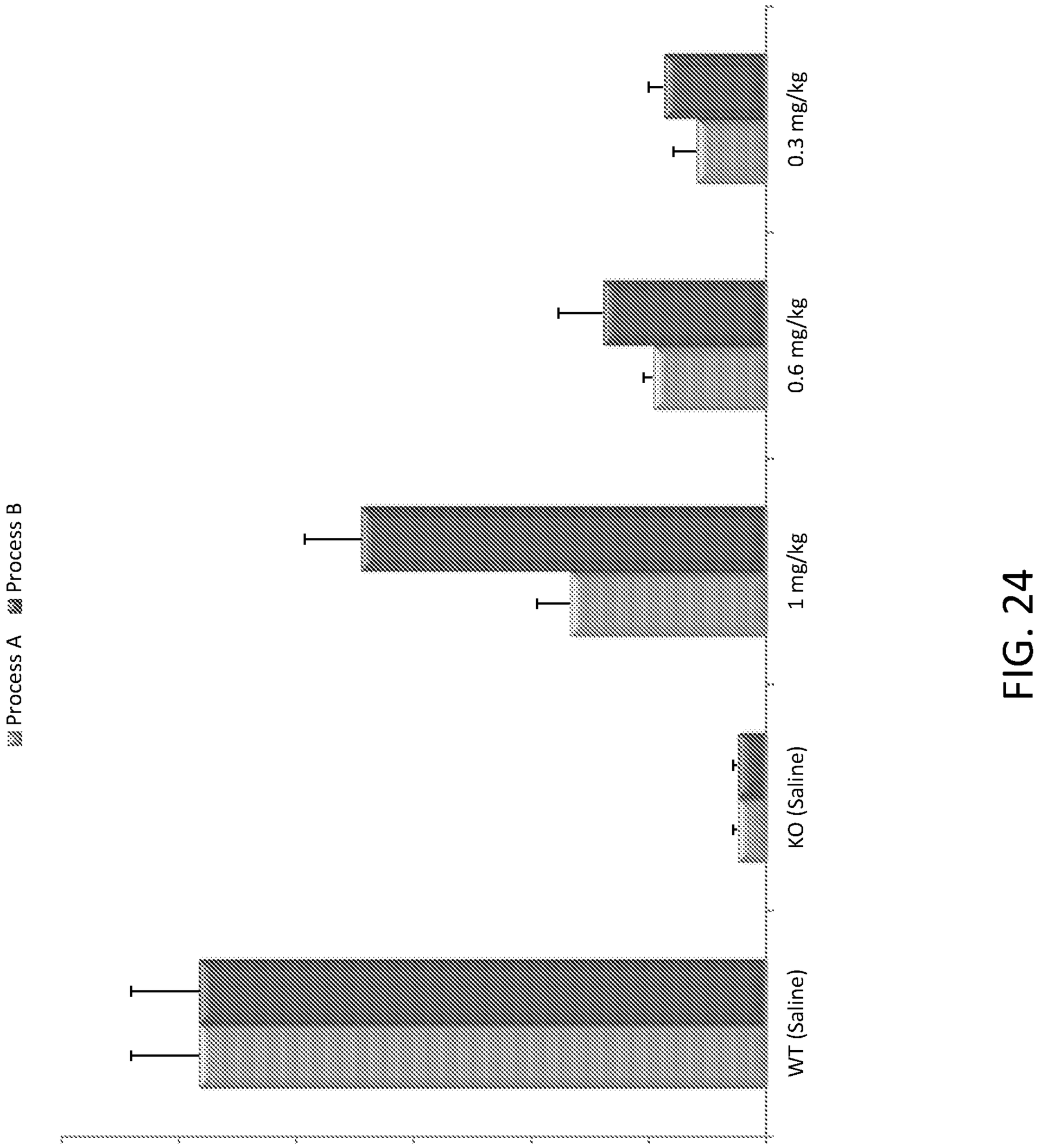

Process A
Process B
1 mg/kg
0.6 mg/kg
0.3 mg/kg
WT (Saline)
KO (Saline)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
umol citrulline/hr/mg total protein

FIG. 24

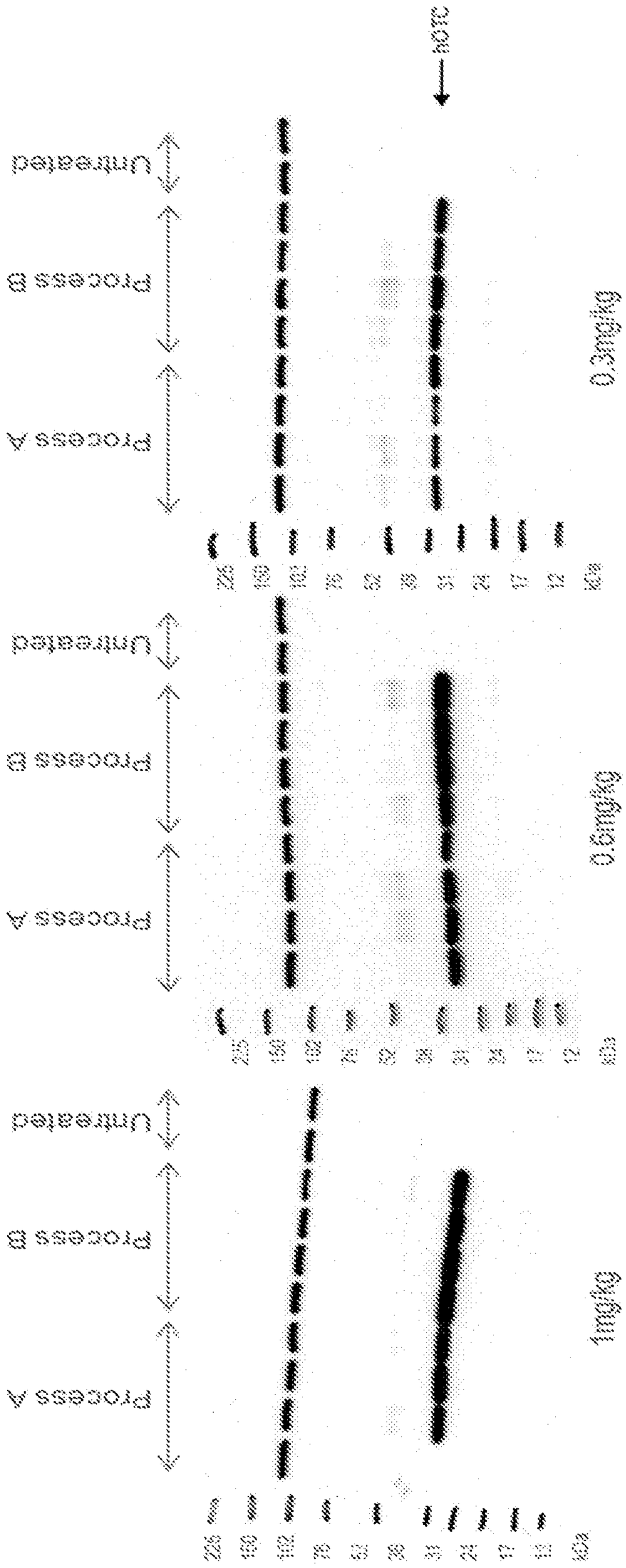

Process A
Process B
Untreated
0.6mg/kg
Process A
Process B
Untreated
0.3mg/kg
Process A
Process B
Untreated
1mg/kg
Process A
Process B
Untreated
Dose

FIG. 25

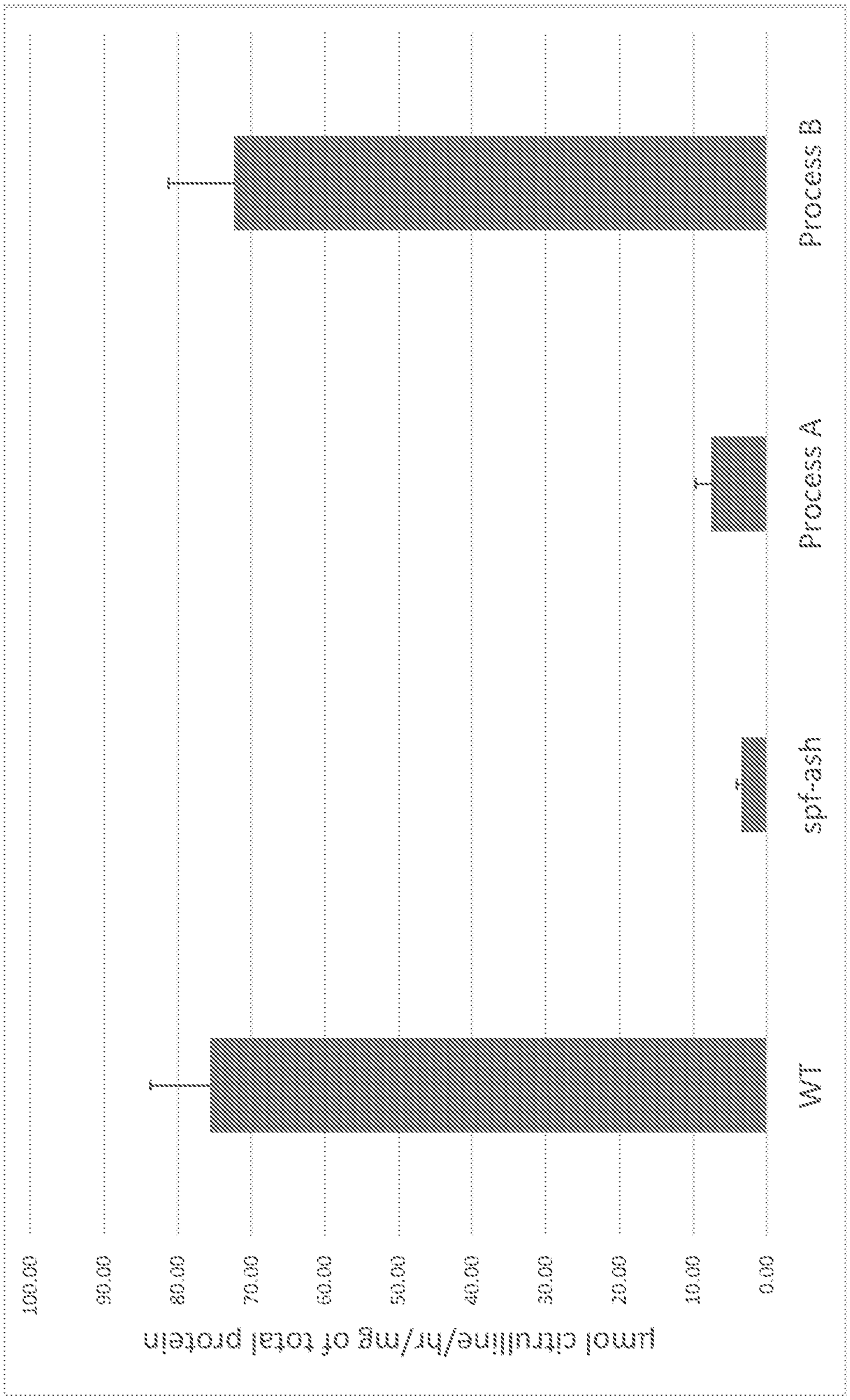

WT
spf-ash
Process A
Process
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
μmol citrulline/hr/mg of total protein

FIG. 26

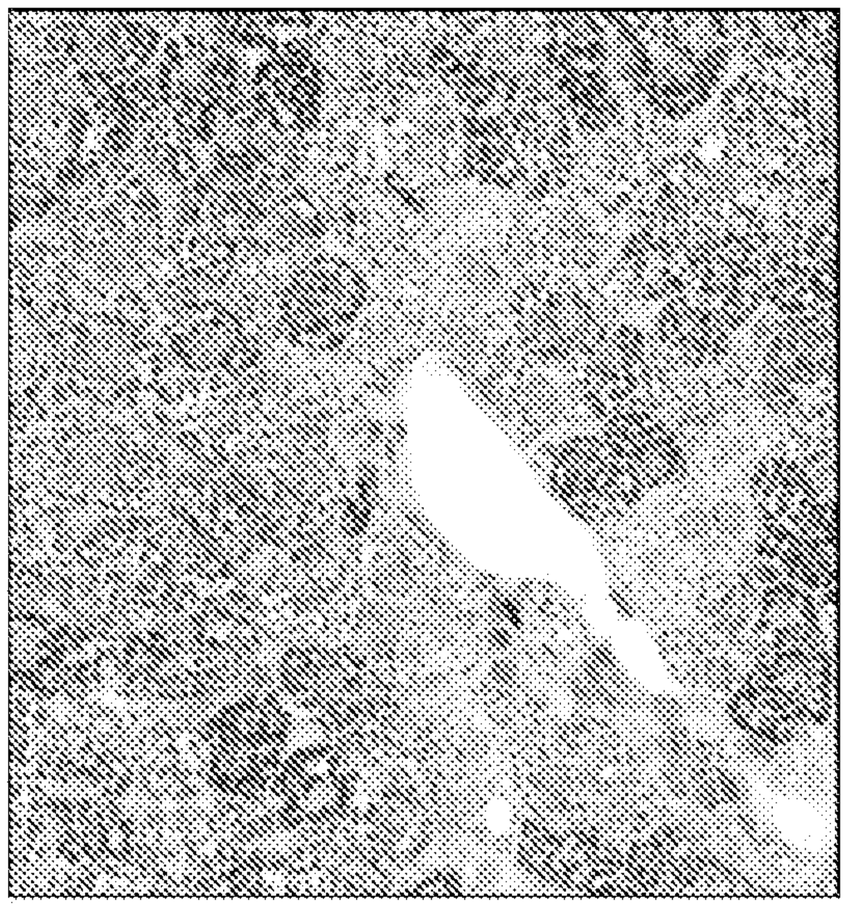


FIG. 27(b)



FIG. 27(d)

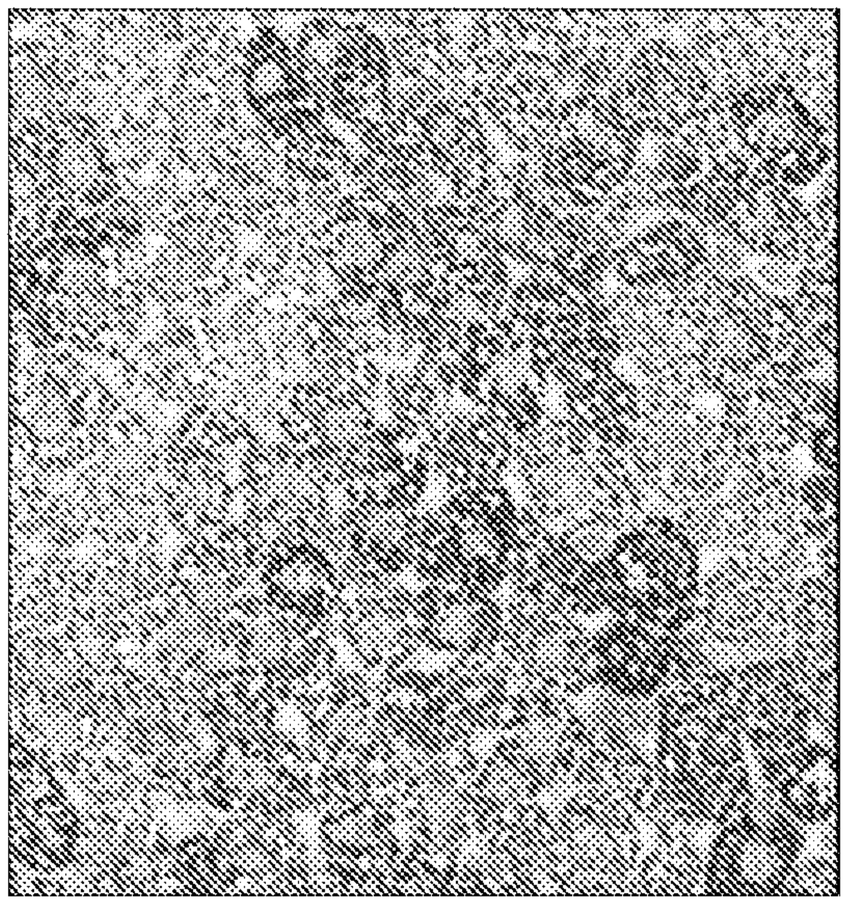


FIG. 27(a)

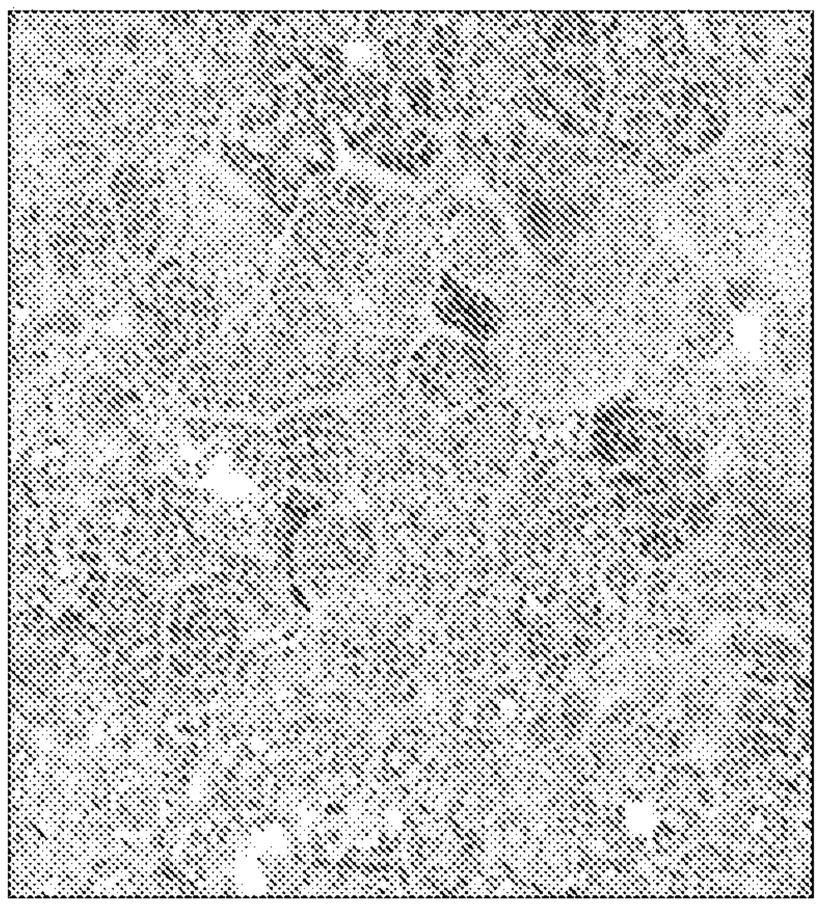


FIG. 27(c)

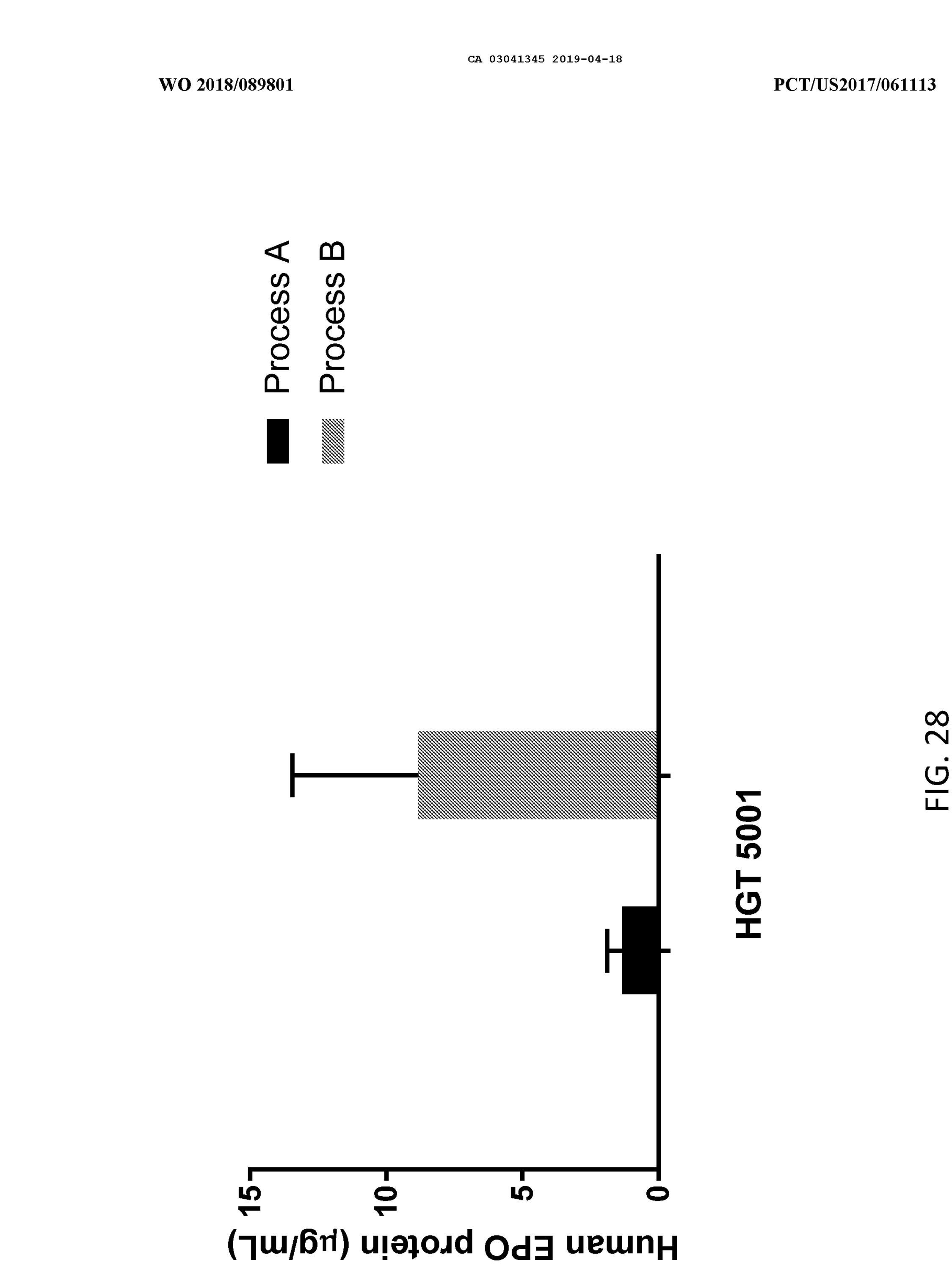


FIG. 28

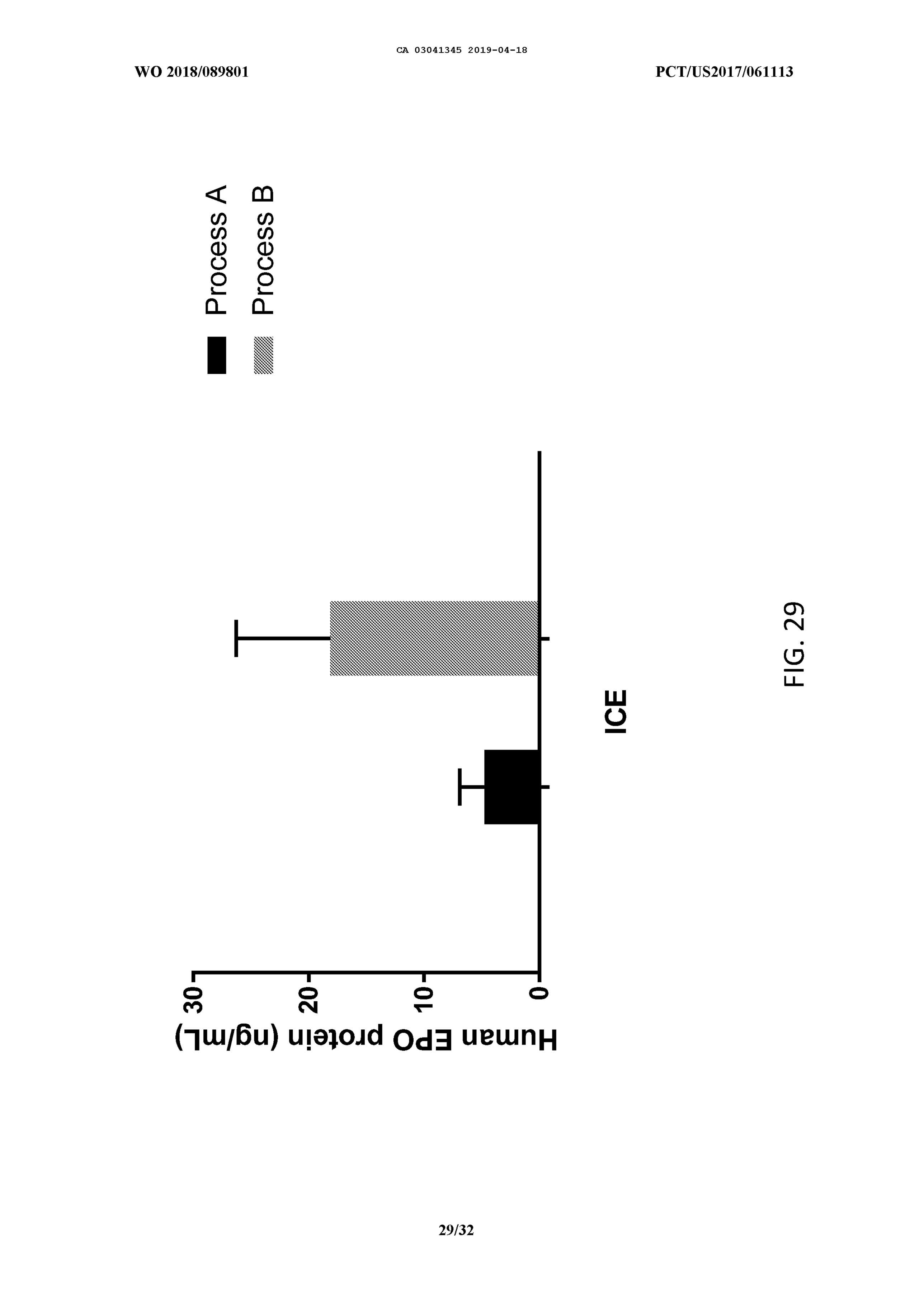


FIG. 29

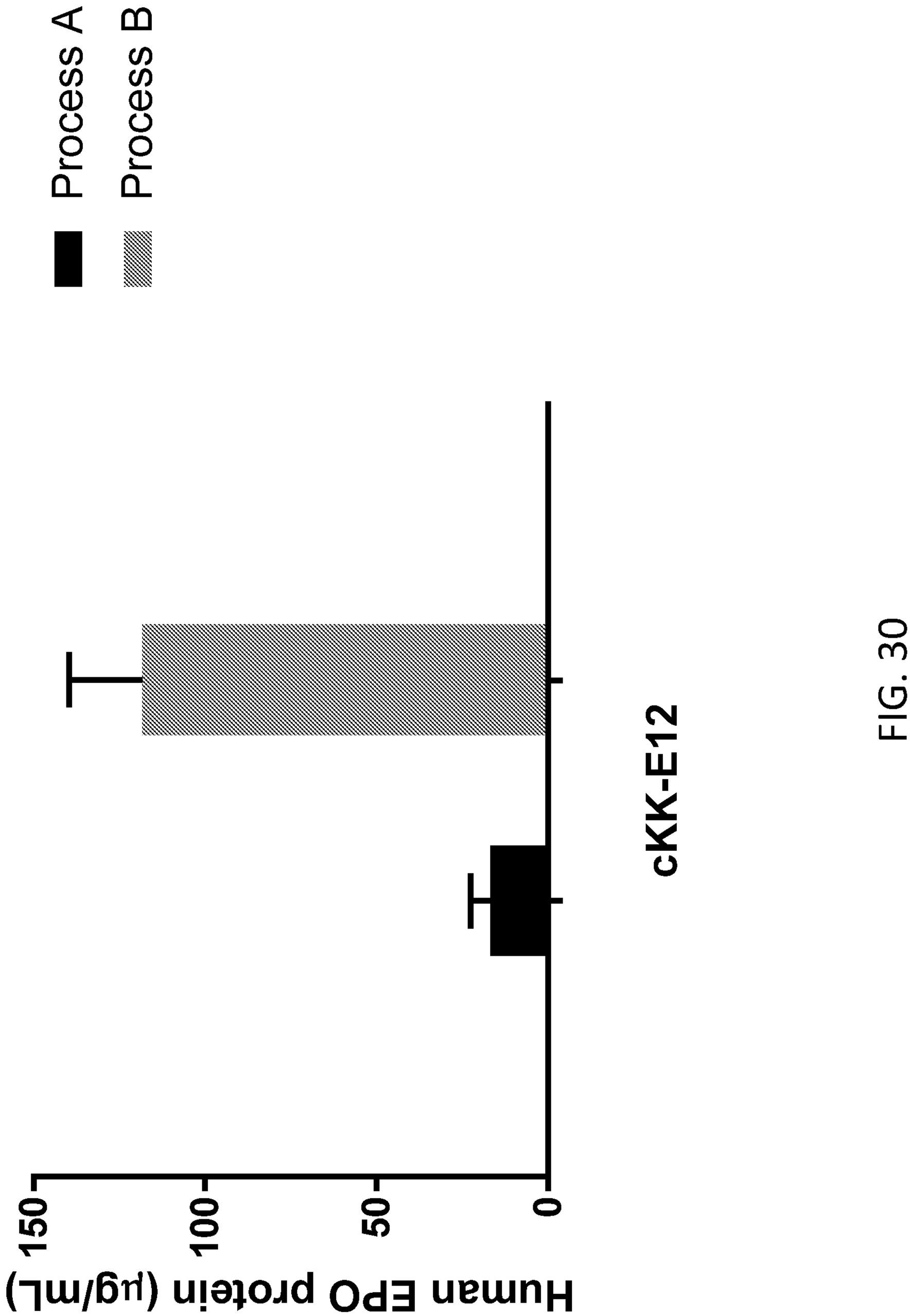

Process A
Process B
cKK-E12
Human EPO protein (µg/mL)
150
100
50
0

FIG. 30

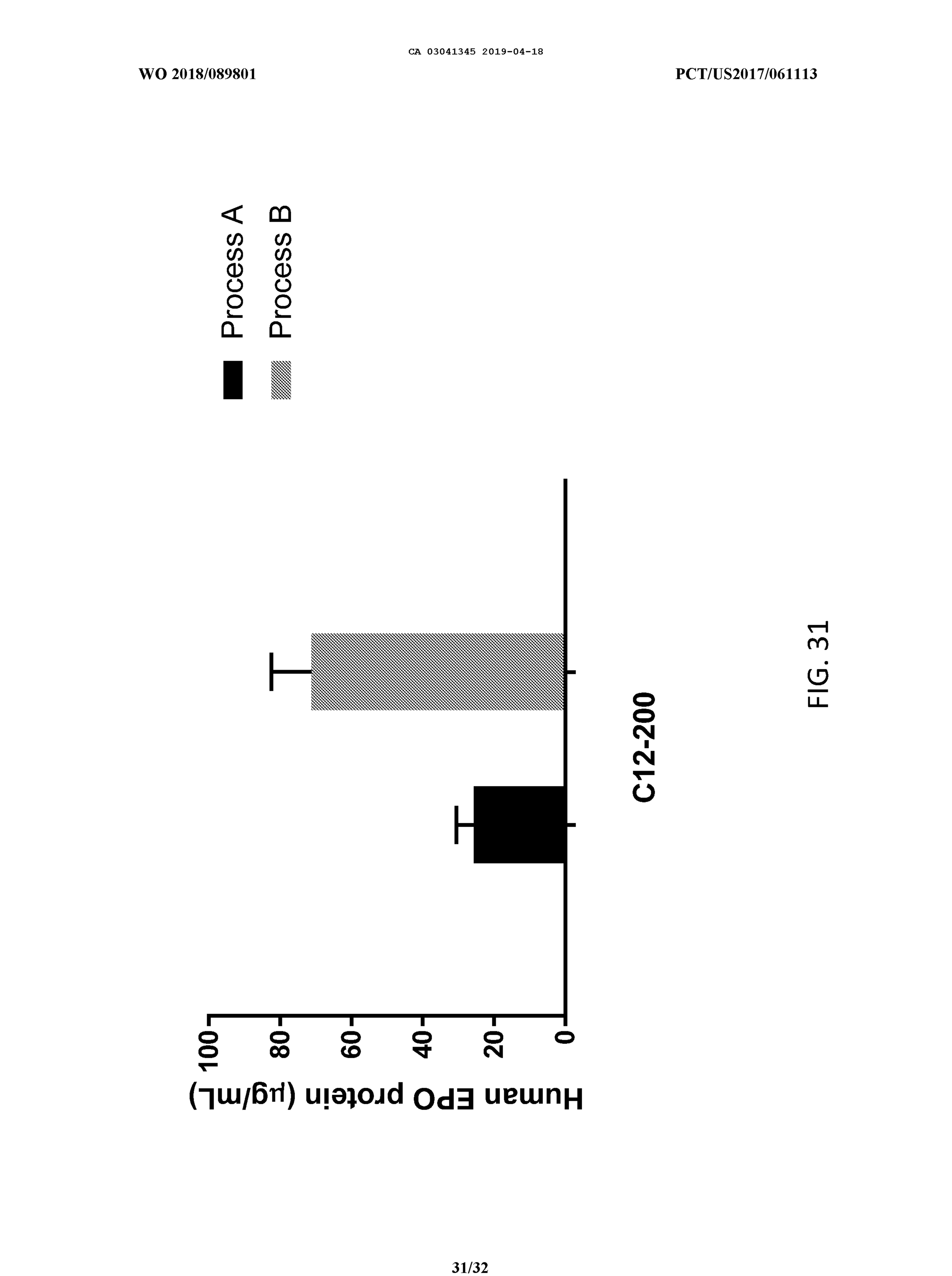

Process A
Process B
C12-200
Human EPO protein (µg/mL)
100
80
60
40
20
0

FIG. 31

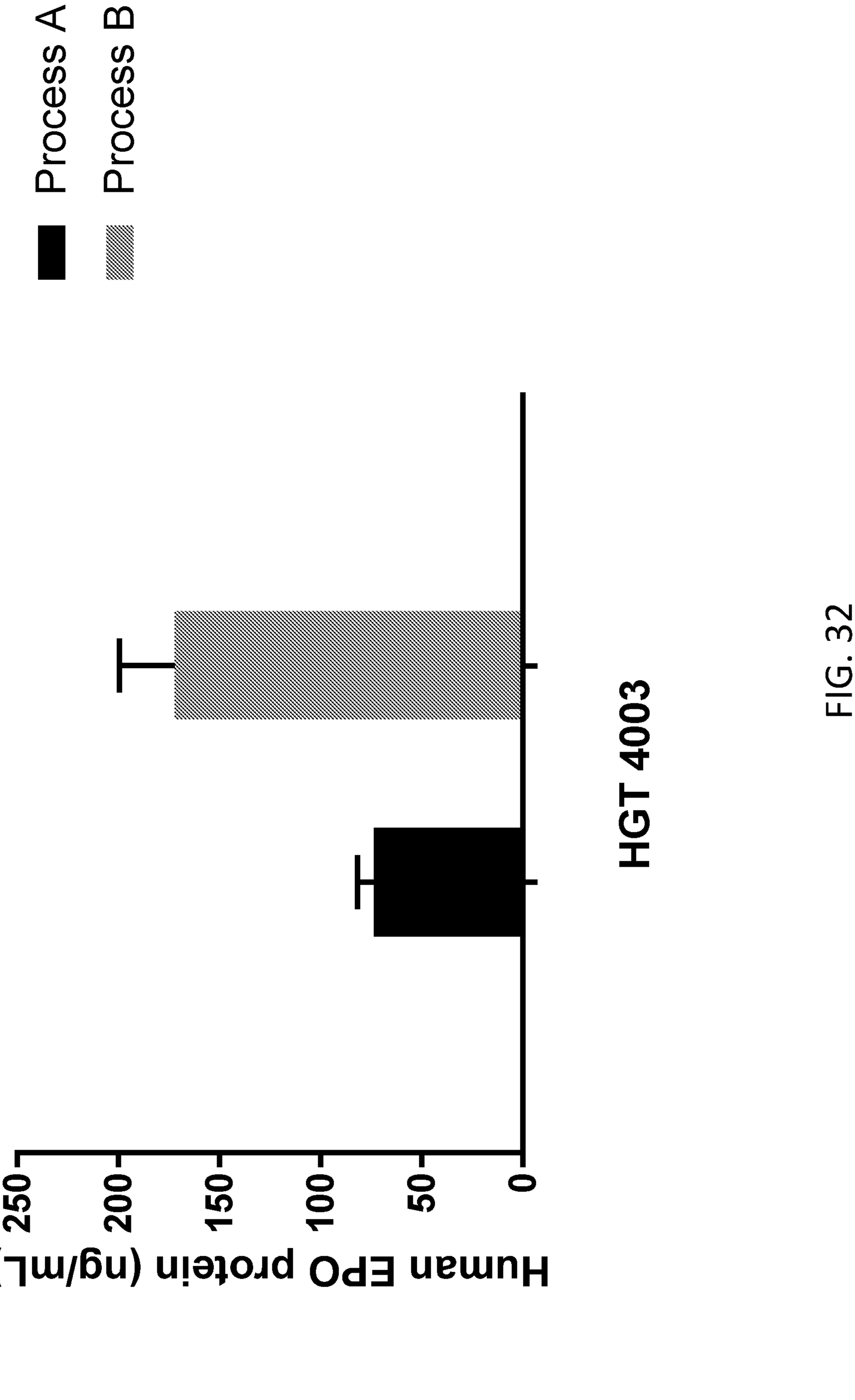

Process A
Process B
HGT 4003
Human EPO protein (ng/mL)
250
200
150
100
50
0

FIG. 32

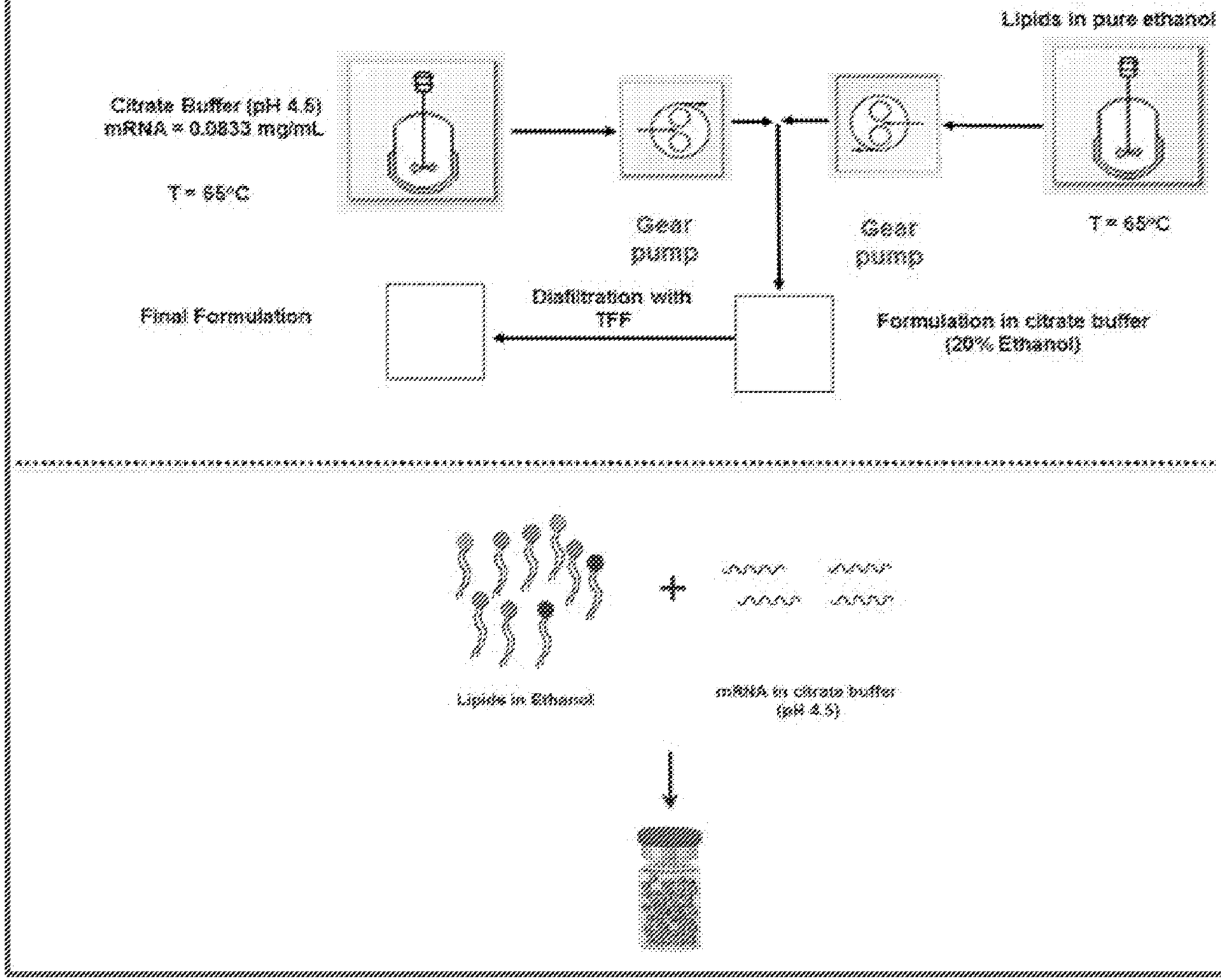

Lipids in pure ethanol
Citrate Buffer (pH 4.5)
mRNA = 0.0833 mg/mL
T = 65°C
Gear pump
Gear pump
T = 65°C
Final Formulation
Diafiltration with TFF
Formulation in citrate buffer (20% Ethanol)
Lipids in Ethanol
mRNA in citrate buffer (pH 4.5)

FIG. 1