[009] The present disclosure provides a cell culture and transient transfection system, whereby the system supports introduction by way of transfection and subsequent expression of one or more macromolecules (such as, e.g., expressible nucleic acids such as a DNA or an RNA molecule containing at least one protein coding region and additional genetic elements that allow for the expression thereof in a host cell under appropriate culturing conditions) into a plurality of eukaryotic CHO cells in suspension culture, and further supports the cultivation and growth of the CHO cells subsequent to the introduction/transfection, wherein growth and/or maintenance of the at least one CHO cell continues in the high density growth medium in the absence of the high density growth medium being supplemented, replenished or replaced with fresh high density growth medium.

[0010] In some examples, it is not necessary to remove, supplement, replenish or replace the high density growth medium used during or after theintroduction/transfection of the CHO cells from the presence of the CHO cells to support the further growth thereof. In another example, after the introduction/transfection, growth of the CHO cells and production of an expressed protein from the expressible nucleic acid can be accomplished in a volume of medium that is about the same volume up to no more than about 50% more than the volume of the high density growth medium in which the introduction/transfection occurred. Using the high density growth medium of the present disclosure, it is not necessary to replenish, replace or supplement the medium after one has introduced nucleic acid into cells, and before cells into which nucleic acid has been introduced are further cultured to transiently express a protein from the nucleic acid.

[0011] Transient expression is fast becoming the system of choice for rapid mammalian protein production. The flexibility of transient transfection enables a rapid realization time from concept to protein-in-hand and many different proteins can be produced simultaneously, or serially. The next key advance in transient transfection technology is to approach or equal expression levels attained using stable expression systems without losing the speed and flexibility of the transient format. We report for the first time the development of a novel transient transfection system that utilizes high density CHO-S cell cultures to generate expression levels of > 2g/L (up to about 4 g/L) of recombinant proteins, such as, e.g., human IgG and non-IgG proteins within 14 days after cells are transfected.

[0012] To attain such high levels of protein expression, a novel cell culture system which includes a high density growth medium in combination with a population of suspension CHO cells that are adapted for high density growth in such a media, and further in combination with a protein expression enhancer composition and a cell growth modulator composition was developed that allows certain populations of CHO cells to reach viable cell densities of up to 40 $x10^6$ cells/ml (more typically up to about $20x10^6$ cells/ml). These high density CHO cultures enable transfection and protein expression at higher cell densities and protein titers than traditional protocols, significantly increasing the volumetric yield of protein. The addition of one or more expression enhancer formulations and one or more growth modulator compositions as described herein following or during the transfection workflow was also found to boost protein expression level to levels up to 10- to 20-fold higher than the expression levels seen with current commercially available transient transfection systems. High expressing clones of suspension CHO cells (hereinafter referred to as CHO-S cells) were selected and thereafter were adapted for improved growth, viability characteristics under high density culture conditions, and the expression system and workflow were then further optimized for increased protein production. The resulting high density CHO-S-H2H cells have an optimized growth rate, increased cell size, reduced cell clumping in suspension, and increased specific productivity compared to the parental CHO-S line or cell lines that gave rise to CHO-S cells. Finally, the transfection method and workflow was optimized through the use of one or more transfections reagents that are used in combination with one or more expression enhancer formulations and one or more growth modulator compositions to further increase overall protein yield such that protein yields in the range of about1 g/L to about 4 g/L of initial culture volume, or any range or concentration range falling therein, are routinely achievable.

[0013] When all of these improvements were combined into a single expression system, protein levels were increased up to 20, 30, 40-fold for both IgG and non-IgG recombinant proteins compared to the commercially available FreeStyle™ 293 or Expi293™ expression system and expression levels of > 2 g/L were attained for multiple proteins. Additionally, protein functionality was demonstrated to be comparable for several proteins expressed in the high yield expression system of the present disclosure when compared to the popular commercially available FreeStyle™ 293 or Expi293™ expression systems. Together, these results indicate that significant increases in functional protein yields can be attained using a

novel transient CHO expression system that incorporates numerous advances in protein expression technology into a single, easy to use format.

[0014] The present disclosure also provides a method of cultivating CHO cells comprising: (a) contacting the cells with the cell high density growth medium of the present disclosure; (b) maintaining the cells under conditions suitable to support cultivation of the CHO cells in culture; and (c) expressing a nucleic acid to form a protein product.

[0015] The present disclosure also provides a method for introducing one or more macromolecules into at least one CHO cell in culture, the method comprising: (a) culturing at least one eukaryotic cell in the high density growth medium in culture; (b) introducing at least one macromolecule into the culture under conditions suitable to cause one or more of the at least one macromolecules to be introduced in the at least one cell; and (c) cultivating the at least one cell in the high density growth medium to produce a product whose production is controlled by the at least one molecule, wherein growth of the at least one CHO cell continues in the high density growth medium in the absence of the high density growth medium being replaced, replenished or supplemented with fresh high density growth medium, wherein it is not necessary to remove high density growth medium used during the introduction from the presence of the at least one CHO cell to support growth thereof, or to support the expression of the protein encoded by the one or more nucleic acids transfected therein, and/or wherein after the introduction, growth is accomplished in a final culture volume that is about the same volume up to no more than about 50% the volume of the culture in which the introduction occurred. In particular any increase in culture volume will preferably be due to the addition of growth modulator compositions or expression enhancer compositions to the culture following transfection of the cells, rather than due to the addition or supplementation of the high density growth medium with fresh high density growth medium.

[0016] The present disclosure also provides kits for the cultivation and transfection of CHO cells in vitro, the kits comprising the high density growth medium of the present disclosure, and optionally further comprising one or more of: one or more agents for the introduction of at least one molecule into a cell, one or more macromolecules, a population of CHO-S cells, e.g., CHO-S-2H2 cells, one or more expression enhancer compositions, one or more growth modulator compositions, as well as instructions for culturing the at least one

CHO cell in culture and/or for introducing at least one macromolecule into at least one CHO cell in culture.

[0017] The present disclosure also provides a composition comprising the CHO cell high density growth medium of the present disclosure and at least one component selected from the group consisting of at least one CHO cell, one or more agents for the introduction of at least one macromolecule into at least one cell (i.e., one or more transfection agents, which may preferably be one or more cationic lipids), one or more expression enhancer compositions, one or more growth modulator compositions, and one or more macromolecules comprising an expressible nucleic acid comprising at least one protein coding region in addition to one or more elements enabling the expression thereof in a host cell under appropriate conditions that allow for protein expression.

[0018] In one aspect, there is provided a method for producing a recombinant protein in cultured suspension CHO cells, said method comprising: obtaining a suspension culture of CHO cells, said CHO cells being adapted for growth under high density culture conditions, and culturing said CHO cells at a cell density of between about $2x10^6$ to about $2x10^7$ cells/ml in a high density growth medium adapted to allow the growth of suspension CHO cells, transfecting said CHO cells with an expression vector in the presence of a transfection reagent, the expression vector comprising a nucleic acid sequence capable of producing an expressed protein; incubating said transfected CHO cells for a first period of time, contacting said transfected CHO cells with at least one expression enhancer composition and at least one growth modulator composition, incubating said transfected CHO cells in the presence of said transfection enhancer and growth modulator for a second period of time under conditions such that said expression vector expresses said protein; and harvesting said transfected CHO cells and isolating the expressed protein.

[0019] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include, after the aforementioned second period of time, contacting the transfected cells a second time with said growth modulator composition and incubating said transfected cells for at least a third period of time prior to harvesting the transfected CHO cells and isolating the expressed protein.

[0020] In some examples, the aforementioned third period of time may be up to about 20 days, up to about 15 days, up to about 14 days, up to about 13 days, up to about 12 days,

up to about 11 days, up to about 10 days, up to about 9 days, up to about 8 days, up to about 7 days, up to about 6 days, up to about 5 days, up to about 4 days, about 20 days, about 15 days, about 14 days, about 13 days, about 12 days, about 11 days, about 10 days, about 9 days, about 8 days, about 7 days, about 6 days, about 5 days, about 4 days.

[0021] In some examples, the transfected cells may be contacted with the aforementioned expression enhancer composition and the growth modulator compositions and then further cultured at a temperature of less than 37°C and greater than 30°C, less than 35°C and greater than 31°C, or at a temperature of about 32°C.

[0022] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include obtaining suspension CHO cells that are CHO-S cells, or a derivative of CHO-S cells, which cells have been adapted for growth under high density culture conditions and which have been selected for increased production of recombinant protein. In some examples, the suspension CHO cells may be CHO-S-2H2 cells, CHO-S-clone 14 cells, or ExpiCHO-S cells.

[0023] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include culturing or cultivating suspension CHO cells in an appropriate high density growth medium at a cell density of between about $3x10^6$ to about $15x10^6$ cells/ml, about $3.5x10^6$ to about $12x10^6$ cells/ml, about $4x10^6$ to about $10x10^6$ cells/ml, about $4.5x10^6$ to about $9x10^6$ cells/ml, about $5x10^6$ to about $8x10^6$ cells/ml, about $5.5x10^6$ to about $7x10^6$ cells/ml, about $6x10^6$ to about $6.5x10^6$ cells/ml, about $6x10^6$ to about $6.25x10^6$ cells/ml, or about $6x10^6$ cells/ml.

[0024] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include obtaining a transfection reagent optimized for use in the presently described protein expression system. In an example, the transfection reagent may be a cationic lipid or a polymeric amine-based transfection reagent. In an example, a suitable transfection reagent may include a polyethylenimine (PEI) polymer, or a derivative thereof, such as, e.g., a linear PEI. In an example, a transfection reagent optimized for use in the presently described protein expression system may include a cationic lipid. A suitable cationic lipid may include one or more cationic lipids of the type disclosed in PCT Publ. No. WO 2007/130073 entitled "Novel Reagents for Transfection of Eukaryotic Cells", PCT Publ. No. WO 2015/089487 entitled "MEMBRANE-PENETRATING PEPTIDES TO ENHANCE

TRANSFECTION AND COMPOSITIONS AND METHODS FOR USING SAME" and PCT Publ. No. WO 2000/027795 entitled "Transfection Reagents", all of which are hereby expressly incorporated by reference in their entirety as though fully set forth herein.

[0025] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include contacting a cationic lipid with an expression vector or a nucleic acid molecule to form a transfection complex prior to transfecting said suspension CHO cells. The cationic lipid may be contacted with the expression vector in a suitable aqueous medium that facilitates the formation of a transfection complex between the cationic lipid and the nucleic acid molecule. In certain non-limiting examples, the transfection complex may formed at a temperature of greater than 0°C and less than 20°C, less than 15°C, less than 10°C, less than 8°C, or less than 5°C.

[0026] Suitable volumes of suspension cultures suitable for use with the examples described herein may include any volume in the range of 10 mL to about 5 L, though it will be readily apparent to one skilled in the art that the methods, systems, kits and reagents contemplated herein are scalable and modification of the system or methods to adapt to unspecified culture are within the skill level of a person having ordinary skill level in the art, and are likely encompassed herein without departing from the spirit and scope of the invention. In some examples, the volume of the suspension culture used in accordance with the present disclosure prior to the transfection of the suspension CHO cells is in the range of about 20 mL to about 1500 ml, about 25 ml to about 1000 ml, about 30 ml to about 750 ml, about 50 ml to about 500 ml, about 75 ml to about 400 ml, about 100 ml to about 200 ml, or any ranges therebetween. In some examples, the volume of the suspension culture used in accordance with the present disclosure prior to the transfection of the suspension CHO cells is about 20 ml, about 25 ml, about 30 ml, about 35 ml, about 40 ml, about 45 ml, about 50 ml, about 55 ml, about 60 ml, about 65 ml, about 70 ml, about 75 ml, about 80 ml, about 100 ml, about 125 ml, about 150 ml, about 175 ml, about 200 ml, about 250 ml, about 300 ml, about 400 ml, about 500 ml, about 750ml, 1000 ml, or about 1500 ml, or any volume therebetween.

[0027] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include contacting the suspension CHO cells with an expression enhancer composition following the transfection thereof with a nucleic acid molecule. In some examples, a suitable expression enhancer composition may include at least one,

optionally more than one, optionally, two, optionally three, optionally four of valproic acid, sodium propionate, sodium butyrate, lithium acetate, dimethyl sulfoxide (DMSO), galactose, amino acids, or any combinations thereof. In some examples, an expression enhancer composition may include at least two, at least three, or at least four or more of valproic acid, sodium propionate, sodium butyrate, lithium acetate, dimethyl sulfoxide (DMSO), galactose, amino acids, or any combinations of the aforementioned. In some examples, an expression enhancer composition used in accordance with the present disclosure may include valproic acid, sodium propionate, and sodium butyrate.

[0028] In some examples, an expression enhancer composition may optionally include a composition that reduces clumping of suspension cells by at least 85%, at least 80%, at least 75%, at least 70%, at least, 65%, at least 60%, at least 55% or at least 50%. Such a composition that reduces clumping of suspension cells may include dextran sulfate. In some examples, an expression enhancer composition may include between about 10 mg/ml to about 200 mg/ml, about 25 mg/ml to about 175 mg/ml, about 50 mg/ml to about 150 mg/ml, about 75 mg/ml to about 125 mg/ml, between about 90 mg/ml to about 110 mg/ml, or any concentration or concentration ranges therebetween.

[0029] In some examples, an expression enhancer composition may include valproic acid (VPA). VPA may be present in an expression enhancer composition. The concentration of valproic acid in the expression enhancer composition may be in the range of about 5 mg/ml to about 50 mg/ml, about 5 mM to about 200 mM, or any concentration therebetween. The concentration of valproic acid in the expression enhancer composition may be in the range of about 20 mM to about 150 mM, about 25 mM to about 125 mM, about 50 mM to about 100 mM, about 75 mM to about 80 mM. The concentration of valproic acid in the expression enhancer composition may be about 50 mM to about 150 mM, about 75 mM to about 125 mM, about 80 mM to about 120 mM, about 90 mM to about 110 mM or about 100 mM. The concentration of valproic acid in the expression enhancer composition may be about 10 mg/ml to about 20 mg/ml, about 12 mg/ml to about 18 mg/ml, about 14 mg/ml to about 16 mg/ml or any concentration therebetween. The concentration of valproic acid in the expression enhancer composition may be about 10 mg/ml, about 10.5 mg/ml, about 11 mg/ml, about 11.5 mg/ml, about 12 mg/ml,, about 12.5 mg/ml, about 13 mg/ml, about 13.5 mg/ml,, about 14 , about 14.5 mg/ml, about 15 mg/ml, about 15.5 mg/ml, about 16 mg/ml,

about 16.5 mg/ml, about 17 mg/ml, about 17.5 mg/ml, about 18 mg/ml, about 18.5 mg/ml, about 19 mg/ml, about 19.5 mg/ml, or about 20 mg/ml, or any concentration therebetween.

[0030] In some examples, an expression enhancer composition may include sodium propionate. When used, the final concentration of sodium propionate in the culture after the expression enhancer composition has been added thereto may be in the range of about 0.2 mM to about 100 mM, about 0.5 mM to about 50 mM, about.0.75 mM to about 25 mM, about 1 mM to about 15 mM, about 1.25 mM to about 10 mM, about 1.5 mM to about 5 mM, about b0.75 mM about 0.8 mM, about 1 mM, about 1.2 mM, about 1.4 mM, about 1.5 mM, about 1.5 mM, about 1.7 mM, about 2.0 mM, or any concentration therebetween. In some examples, the final concentration of sodium propionate in the culture after the expression enhancer composition has been added thereto may be range of about 0.1 mg/ml to about 0.2 mg/ml, or any concentration therebetween. In some examples, the final concentration of sodium propionate in the culture after the expression enhancer composition has been added thereto may be about 0.5 mM to about 5 mM, or any concentration therebetween.

[0031] In some examples, the final concentration of sodium propionate in the culture after the expression enhancer composition has been added thereto may be range of about 0.01 mM to about 1 mM, about 0.05 mM to about 0.5 mM, about 0.01 mM to about 0.25 mM, about 5 mg/L to about 20 mg/L, about 8 mg/L to about 15 m/L, about 10 mg/L to about 14 mg/L, or any concentration therebetween.

[0032] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include obtaining a suspension culture of CHO cells having a volume in the range of about 25 mL to about 50 L, or any concentration therebetween. In some examples, the culture volume may be in the range of about 100 mL µl to about 1 L. In some examples, the volume of the suspension culture may be in the range of about 200 mL µl to about 500 ml.

[0033] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include obtaining a suspension culture of CHO cells having the cell density of the transfection step is between about $1x10^6$ to about 50 $x10^6$ cells/ml, about $2x10^6$ to about $25x10^6$, about $10x10^6$ to about $20x10^6$ cells/ml, or any cell density or range of cell densities falling therein.

[0034] In some examples, methods for producing a recombinant protein in cultured suspension CHO cells according to the present disclosure may include culturing the cell for a prolonged period, e.g., for up to 20 days, following the transfection thereof in the high density growth medium in which they were transfected, and maintaining the cells therein under conditions permissive to the expression of a recombinant protein, and further without requiring that the high density growth medium be replaced, replenished, or supplemented with additional or fresh medium following the transfection step. In some examples, the high density growth medium is not replaced, replenished or supplemented following the transfection step following the transfection of the cells with an expressible nucleic acid, though in some examples the post-transfection culture may be supplemented with up to 30%, 40% or up to 50% of the initial culture volume with a growth modulator composition as defined herein.

[0035] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include obtaining a high density growth medium that is a serum-free/protein-free chemically defined culture medium that promotes the growth of transfected CHO cells at densities in excess of 2×10^6 cells/ml up to about 5×10^7 cells/ml with cell viability remaining in excess of 75%, 80%, 85%, 90%, 95% and with yields of recombinant protein of at least 1 mg/ml, 1.25 mg/ml, 1.5 mg/ml, 1.75 mg/ml, 2 mg/ml, 2.25 mg/ml, 2.5 mg/ml, 2.75 mg/ml, 3 mg/ml, 3.25 mg/ml, 3.5 mg/ml, 3.75 mg/ml, up to 4 mg/ml of initial cell culture volume.

[0036] In some examples, a method for producing a recombinant protein in cultured suspension CHO cells may include obtaining a high density growth medium that is a serum-free/protein-free chemically defined culture medium that promotes the growth of transfected CHO cells at densities in excess of 2×10^6 cells/ml to about 5×10^7 cells/ml with cell viability remaining in excess of 75%, 80%, 85%, 90%, 95% after the transfection thereof with said expression vector.

[0037] In some examples, methods for producing a recombinant protein in cultured suspension CHO cells according to the present disclosure may include culturing the cell for a prolonged period, e.g., for up to 20 days, following the transfection thereof in the high density growth medium in which they were transfected, and maintaining the cells therein under conditions permissive to the expression of a recombinant protein, and

further without requiring that the high density growth medium be replaced, replenished, or supplemented with additional or fresh medium following the transfection step.

[0038] In some examples, methods for producing a recombinant protein in cultured suspension CHO cells according to the present disclosure may include culturing the cell for a prolonged period, e.g., for up to 20 days, following the transfection thereof in the high density growth medium in which they were transfected, and maintaining the cells therein under conditions permissive to the expression of a recombinant protein, and further where the high density growth medium is not supplemented, replaced, or replenished following the transfection step.

[0039] In some examples, methods for producing a recombinant protein in cultured suspension CHO cells according to the present disclosure may include transfecting the suspension CHO cells with an expressible nucleic acid and culturing the CHO under conditions permissive to the expression of a recombinant protein from the expressible nucleic acid for at least a first period of time, the first period of time being in the range of about 12 hrs to about 2 days, about 15 to about 36 hrs, about 16 hrs to about 30 hrs, about 18 to about 28 hrs, about 19 to about 26 hrs, about 19 to about 25 hrs, about 20 to about 24 hrs, or any time therebetween. In some examples, the first period of time may be about 15 hrs, about 16 hrs, about 17 hrs, about 18 hrs, about 19 hrs, about 20 hrs, about 21, hrs, about 22 hrs, about 23 hrs, about 24 hrs, about 25 hrs, about 26 hrs, about 27 hrs, about 28 hrs, up to 48 hrs, or any time therebetween.

[0040] In some examples, methods for producing a recombinant protein in cultured suspension CHO cells according to the present disclosure may include transfecting the suspension CHO cells with an expressible nucleic acid and culturing the CHO under conditions permissive to the expression of a recombinant protein from the expressible nucleic acid for a first period of time, and contacting the transfected cells with at least an expression enhancer composition and a at least a growth modulator composition for a second period of time, the second period of time being up to about 20 days, up to about 19 days, up to about 18 days, up to about 17 days, up to about 16 days, up to about 15 days, up to about 14 days, up to about 13 days, up to about 12 days, up to about 11 days, up to about 10 days, up to about 9 days, up to about 8 days, up to about 7 days, up to about 6 days, up to about 5 days, up to about 4 days, up to about 3 days, or any time therebetween.

[0041] In some examples, a growth modulator composition may include glucose. In some examples, the osmolality of glucose in said growth modulator composition may be between about 500 mOsm/kg to about 700 mOsm/kg, about 550 mOsm/kg to about 650 mOsm/kg, about 575 mOsm/kg to about 625 mOsm/kg. In some examples, concentration of glucose in the growth modulator may be in the range of about 85 mg/ml to about 115 mg/ml, about 90 mg/ml to about 110 mg/ml, about 95 mg/ml to about 105 mg/ml.

[0042] In some examples, a growth modulator composition may include a plurality of amino acids, each amino acids having a concentration in the range of about 0.1 mg/ml to about 8 mg/ml. In some examples, a growth modulator composition may have an osmolality of between about 1000 mOsm/kg to about 1500 mOsm/kg, about 1100 mOsm/kg to about 1400 mOsm/kg, about 1200 mOsm/kg to about 1300 mOsm/kg, or any osmolality or range therebetween. In some embodiments, a growth modulator composition may have an osmolality of about 1100 mOsm/kg, about 1150 mOsm/kg, about 1200 mOsm/kg, about 1250 mOsm/kg, about 1300 mOsm/kg, about 1350 mOsm/kg, about 1400 mOsm/kg, about 1450 mOsm/kg, about 1500 mOsm/kg.

[0043] In some examples, the volume of the culture when the transfected CHO cells are harvested may be less than about 150%, less than about 145%, less than about 140%, less than about 135%, less than about 130%, less than about 125%, less than about 120% of the volume of the culture at the time the suspension cells were transfected, where the increase in volume is not due to the addition, supplementation, or replacement of growth medium, but rather is due to the addition of the growth modulator and expression enhancer compositions.

[0044] In some examples, the volume of the culture when the transfected CHO cells are harvested may be less than 150%, less than about 145%, less than about 140%, less than about 135%, less than about 130%, less than about 125%, less than about 120% the volume of the culture at the time the suspension cells were transfected, wherein the increase in volume is due to the addition of the growth enhancer and the expression enhancer compositions.

[0045] Other embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

WHAT IS CLAIMED IS:

1. A method for producing a recombinant protein in cultured suspension CHO cells, said method comprising:

obtaining a suspension culture of CHO cells, said CHO cells being adapted for growth under high density culture conditions,

culturing said CHO cells at a cell density of between about $2x10^6$ to about $2x10^7$ cells/ml in a high density growth medium adapted to allow the growth of suspension CHO cells;

transfecting said CHO cells with an expression vector in the presence of a transfection reagent, said expression vector comprising a nucleic acid sequence capable of producing an expressed protein;

incubating said transfected CHO cells for a first period of time;

contacting said transfected CHO cells with at least one expression enhancer composition and at least one growth modulator composition;

incubating said transfected CHO cells in the presence of said transfection enhancer and growth modulator for a second period of time under conditions such that said expression vector expresses said protein; and

harvesting said transfected CHO cells and isolating the expressed protein.

2. The method according to claim 1, wherein after said second period of time, contacting said transfected cells a second time with said growth modulator composition and incubating said transfected cells for a third period of time prior to harvesting said transfected CHO cells and isolating the expressed protein.

3. The method according to claim 2, wherein said third period of time is up to about 20 days, up to about 15 days, up to about 14 days, up to about 13 days, up to about 12 days, up to about 11 days, up to about 10 days, up to about 9 days, up to about 8 days, up to about 7 days, up to about 6 days, up to about 5 days, up to about 4 days, about 20 days, about 15 days, about 14 days, about 13 days, about 12 days, about 11 days,