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SANDOZ INC.

UNITED STATES DISTRICT COURT

NORTHERN DISTRICT OF CALIFORNIA

SANDOZ INC., Plaintiff, v. AMGEN INC. and AMGEN MANUFACTURING, LIMITED, Defendants.	Case No. **COMPLAINT FOR DECLARATORY JUDGMENT OF PATENT NONINFRINGEMENT AND INVALIDITY**

1 Sandoz Inc. (“Sandoz”) hereby alleges for its Complaint against defendants Amgen Inc.
2 and Amgen Manufacturing, Limited (collectively, “Amgen”), as follows:

3 **NATURE OF THE ACTION**

4 1. This is an action for declaratory judgment of noninfringement and invalidity of
5 U.S. Patent No. 9,643,997 (“the ’997 patent”) arising under the Declaratory Judgment Act,
6 28 U.S.C. §§ 2201-2202, and the patent laws of the United States, 35 U.S.C. § 1 *et seq*.

7 2. This case is a continuation of an already resolved patent dispute venued in this
8 Court involving the same products and the same purification procedures. The same parties
9 litigated patent infringement claims in this District regarding the same patent family, the same
10 products, and the same accused purification processes in Case Nos. 3:14-cv-04741-RS & 3:16-cv-
11 02581-RS. While those cases were pending, Sandoz invited Amgen to include the ’997 patent as
12 a part of those cases and resolve all proceedings in the prior action. However, Amgen did not
13 amend to add those claims in the then-pending litigation; indeed, it did not even respond to
14 Sandoz’s correspondence regarding the patent. Amgen’s claims regarding the related patent with
15 respect to the same products and the same purification procedures were resolved by an order
16 granting judgment under Rule 56 on December 19, 2017. Sandoz believes that elements of that
17 ruling resolve the present dispute as well.

18 3. An actual and justiciable controversy exists under 28 U.S.C. §§ 2201-2202
19 between Sandoz and Amgen as to whether Sandoz infringes any valid claim of the ’997 patent.

20 **PARTIES**

21 4. Sandoz is a corporation organized and existing under the laws of the state of
22 Colorado, with its principal place of business at 100 College Road West, Princeton, New Jersey
23 08540.

24 5. On information and belief, Amgen Inc. is a corporation existing under the laws of
25 the state of Delaware, with its principal place of business at One Amgen Center Drive, Thousand
26 Oaks, California 91320.

27

28

6. On information and belief, Amgen Manufacturing, Limited ("AML") is a corporation existing under the laws of Bermuda, with its principal place of business in Juncos, Puerto Rico 00777.[1]

JURISDICTION AND VENUE

7. This action arises under the Declaratory Judgment Act, 28 U.S.C. §§ 2201-2202, and under the patent laws of the United States, 35 U.S.C. § 1 *et seq*.

8. This Court has subject matter jurisdiction over this action under 28 U.S.C. §§ 1331, 1338(a), and 2201(a).

9. Amgen has previously and voluntarily submitted to jurisdiction and venue in this District with respect to the present patent family and the present products by filing suit in Case Nos. 3:14-cv-04741-RS & 3:16-cv-02581-RS. Amgen has waived any objections to jurisdiction and venue.

10. This Court also has personal jurisdiction over Amgen Inc. because, among other things, Amgen Inc. has continuous and systematic contacts with the State of California, including maintaining its headquarters and multiple facilities in California, including a facility in this District at 1120 Veterans Boulevard, South San Francisco, CA 94080. Amgen Inc. purposefully availed itself of the privileges and protections of this District by engaging in business here, including activities related to patent enforcement. Amgen Inc. previously filed suit against Sandoz in the Northern District of California in Case No. 3:14-cv-04741-RS and again in Case No. 3:16-cv-02581-RS.

11. This Court has personal jurisdiction over AML because, among other things, AML is a wholly owned subsidiary of Amgen Inc. with continuous and systematic contacts with the State of California, including manufacturing pharmaceutical products, including filgrastim and pegfilgrastim, for sale in California. AML purposefully availed itself of the privileges and protections of this District by engaging in business here, including activities related to patent

[1] Although Sandoz does not believe that AML has standing to assert the '997 patent, Amgen nonetheless included AML as a plaintiff in the related prior litigation. This Complaint includes AML to avoid any claim by Amgen that Sandoz did not join all required parties.

enforcement. AML previously filed suit against Sandoz in the Northern District of California in Case No. 3:14-cv-04741-RS and again in Case No. 3:16-cv-02581-RS.

12. Venue in this District is proper under 28 U.S.C. §§ 1391(b) and 1391(c). Defendant Amgen Inc. is subject to personal jurisdiction in this District and resides in this District. Defendant AML may be sued in any district, pursuant to 28 U.S.C. § 1391(c)(3), as a non-resident. Venue is also proper because a substantial part of the events giving rise to the claims in this action occurred in this District.

13. This dispute should be resolved in this District, where venue is proper and where the parties have already litigated similar claims involving the same products, the same patent family, and the same purification processes.

INTRADISTRICT ASSIGNMENT

14. Pursuant to Civil L.R. 3-2(c) and 3-5(b), this is an Intellectual Property Rights Action subject to assignment on a district-wide basis.

15. Pursuant to Civil L.R. 3-12, this case is related to Case Nos. 3:14-cv-04741-RS and 3:16-cv-02581-RS and should be assigned to the same District Judge as those cases.

PATENT-IN-SUIT

16. The '997 patent, entitled "Capture purification processes for proteins expressed in a non-mammalian system," states on its face that it issued on May 9, 2017. A true and correct copy of the '997 patent is attached as Exhibit A.

17. The '997 patent arises from the same set of patent applications that were the source of United States Patent No. 8,940,878 ("the '878 patent"), which was the subject of Case Nos. 3:14-cv-04741-RS and 3:16-cv-02581-RS. The '997 patent stems from U.S. Patent Application No. 12/822,990, which was the application from which the '878 patent issued. Both patents have the same name, have identical abstracts, and share the same specification.

FACTUAL BACKGROUND

18. Sandoz is a global leader in generic and biosimilar medicines, committed to playing a leading role in driving access to medicine worldwide. As set forth in its Mission and Purpose Statement, Sandoz discovers new ways to improve and extend people's lives. Sandoz

contributes to society's ability to support growing healthcare needs by pioneering novel approaches to help people around the world access high-quality medicine.[2]

19. This case concerns filgrastim and pegfilgrastim, biological products approved by the FDA to address certain side effects of cancer treatment. Amgen began selling filgrastim under the brand name Neupogen® in 1991. Amgen's "material U.S. patents for filgrastim (NEUPOGEN®) expired in December 2013."[3] Sandoz obtained approval for its biosimilar filgrastim product, Zarxio, in March 2015 and launched the product in September 2015.

20. A chemical compound known as polyethylene glycol ("PEG") can be attached to filgrastim to create pegfilgrastim. This modification causes the protein to remain in the circulatory system for a substantially longer time, and thus pegfilgrastim is often referred to as "long acting" filgrastim. Amgen began selling pegfilgrastim under the brand name Neulasta® in 2002. Amgen's "final material U.S. patent for Neulasta® expired in October 2015."[4] Sandoz's application to market a biosimilar pegfilgrastim product is currently pending before the FDA.

21. The parties have already litigated patent infringement claims regarding Sandoz's biosimilar filgrastim and pegfilgrastim products in this District. (*See Amgen Inc. v. Sandoz Inc.*, Case Nos. 3:14-cv-04741-RS and 3:16-cv-02581-RS.) Amgen accused a purification step in Sandoz's manufacturing process for filgrastim and pegfilgrastim of infringing U.S. Patent No. 8,940,878 ("the '878 patent"). On December 19, 2017, this Court granted summary judgment of noninfringement of the '878 patent. The Court entered final judgment in favor of Sandoz on January 8, 2018.

22. The '997 patent at issue here and the previously litigated '878 patent are in the same patent family and are similar or identical in several key respects. The alleged inventors are the same. The patent names are the same. The abstracts are the same. Aside from formatting

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[2] *See* Sandoz, Our Mission and Purpose, (last visited February 21, 2019), https://www.sandoz.com/about-us/who-we-are/our-mission-and-purpose.

[3] Amgen Form 10-K for the fiscal year ended December 31, 2013, at 3.

[4] Amgen Form 10-K for the fiscal year ended December 31, 2018, at 47.

and related application data, the specifications of both patents are identical. The ’997 patent issued from a division of the application that issued as the ’878 patent.

23. The claims of both patents are also similar. Claim 9 of the ’997 patent, one of just two independent claims in the ’997 patent, states:

> 9. A method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system comprising:
> (a) solubilizing the expressed protein in a solubilization solution comprising one or more of the following:
> (i) a denaturant;
> (ii) a reductant; and
> (iii) a surfactant;
> (b) forming a refold solution comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of the following:
> (i) a denaturant;
> (ii) an aggregation suppressor;
> (iii) a protein stabilizer; and
> (iv) a redox component;
> (c) applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix;
> (d) washing the separation matrix; and
> (e) eluting the protein from the separation matrix.

By comparison, claim 7 of the ’878 patent, the only independent claim that Amgen asserted in the prior litigation, states:

> 7. A method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system comprising:
> (a) expressing a protein in a non-native limited solubility form in a non-mammalian cell;
> (b) lysing a non-mammalian cell;
> (c) solubilizing the expressed protein in a solubilization solution comprising one or more of the following:
> (i) a denaturant;
> (ii) a reductant; and
> (iii) a surfactant;
> (d) forming a refold solution comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of the following:
> (i) a denaturant;
> (ii) an aggregation suppressor;
> (iii) a protein stabilizer; and
> (iv) a redox component;
> (e) directly applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix;
> (f) washing the separation matrix; and
> (g) eluting the protein from the separation matrix, wherein the separation matrix is a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

1 24. The only differences between claim 9 of the ’997 patent and claim 7 of the ’878
2 patent are shown above in underlined red text. None of those differences are implicated by the
3 grounds on which Sandoz sought summary judgment of noninfringement of the ’878 patent in the
4 prior litigation, which focused on the absence in the accused process of the claimed steps of
5 “washing the separation matrix” and “eluting the protein from the separation matrix.” Those
6 same steps are required by the claims of the ’997 patent, and therefore the same grounds for
7 noninfringement apply with respect to the ’997 patent.

8 25. The ’997 patent issued on May 9, 2017, while the litigation over the ’878 patent
9 was still pending before this Court. On June 7, 2017, Amgen’s counsel sent a letter to Sandoz’s
10 counsel stating Amgen’s belief that it could reasonably assert a claim for infringement of the ’997
11 patent with respect to Sandoz’s making, using, offering to sell, selling, or importing into the
12 United States Sandoz’s filgrastim and pegfilgrastim products.

13 26. In response on July 7, 2017, Sandoz identified the overlap between the pending
14 litigation on the ’878 patent and any potential issues with respect to the ’997 patent. To provide
15 for a prompt and efficient resolution of these issues, Sandoz invited Amgen to bring its purported
16 claims regarding the ’997 patent immediately and resolve them as part of the existing litigation.

17 27. Despite Sandoz’s invitation, Amgen did not amend its claim in the then-pending
18 litigation to assert the ’997 patent and did not otherwise pursue the assertions made in its June 7,
19 2017 letter in any way.

20 28. More than 20 months have passed since Amgen first asserted its potential claims
21 with respect to the ’997 patent, and Amgen still has not filed suit on those claims. Sandoz has
22 been marketing and selling its biosimilar filgrastim product, Zarxio, throughout this time. And as
23 Sandoz notified Amgen on February 21, 2019 pursuant to 42 U.S.C. § 262(*l*)(8)(A), Sandoz
24 intends to begin commercially marketing its biosimilar pegfilgrastim product at the earliest
25 possible opportunity on or after 180 days from the date of its notice.

26 29. Amgen’s delay alone undercuts any claim that Amgen is entitled to any injunctive
27 relief based on the ’997 patent. Nonetheless, Amgen has not disavowed the intention to seek a
28 preliminary injunction pursuant to 42 U.S.C. § 262(*l*)(8)(B). With the filing of this Complaint,

Sandoz seeks to ensure that any issues with respect to the ’997 patent, including any preliminary injunction motion, are resolved promptly, efficiently, and well in advance of the launch of Sandoz’s pegfilgrastim product.

COUNT I
(Declaratory Judgment of Noninfringement of U.S. Patent No. 9,643,997)

30. Sandoz incorporates by reference each allegation set forth in paragraphs 1 through 29 above as if fully set forth herein.

31. Amgen has asserted that Amgen Inc. is the owner of the ’997 patent.

32. Amgen has asserted that the making, use, offer for sale, sale, or importation into the United States of Sandoz’s biosimilar filgrastim and pegfilgrastim products infringes or will infringe the ’997 patent.

33. Sandoz asserts that the making, use, offer for sale, sale, or importation into the United States of Sandoz’s biosimilar filgrastim and pegfilgrastim products does not and will not infringe any valid claim of the ’997 patent, whether directly or indirectly, either literally or under the doctrine of equivalents.

34. As a result of Amgen’s allegations against Sandoz, an actual and justiciable case or controversy exists between Sandoz and Amgen as to the infringement of the claims of the ’997 patent.

35. Sandoz is entitled to a declaration that the making, use, offer for sale, sale, or importation into the United States of Sandoz’s biosimilar filgrastim and pegfilgrastim products does not and will not infringe any valid claim of the ’997 patent. Such a declaration is necessary and appropriate at this time to determine the rights and obligations of the parties.

COUNT II
(Declaratory Judgment of Invalidity of U.S. Patent No. 9,643,997)

36. Sandoz incorporates by reference each allegation set forth in paragraphs 1 through 35 above as if fully set forth herein.

37. Amgen has asserted that Amgen Inc. is the owner of the ’997 patent.

38. Amgen has asserted that the making, use, offer for sale, sale, or importation into the United States of Sandoz's biosimilar filgrastim and pegfilgrastim products infringes or will infringe the '997 patent.

39. Sandoz asserts that the claims of the '997 patent are invalid under one or more provisions of 35 U.S.C. §§ 101, 102, 103, or 112, or other judicially created bases for invalidity.

40. As a result of Amgen's allegations against Sandoz, an actual and justiciable case or controversy exists between Sandoz and Amgen as to the validity of the claims of the '997 patent.

41. Sandoz is entitled to a declaration that the claims of the '997 patent are invalid under one or more provisions of 35 U.S.C. §§ 101, 102, 103, or 112, or other judicially created bases for invalidity. Such a declaration is necessary and appropriate at this time to determine the rights and obligations of the parties.

PRAYER FOR RELIEF

WHEREFORE, Sandoz requests that the Court enter judgment in its favor and against Amgen as follows:

(a) Declaring that Sandoz has not infringed and will not infringe, directly or indirectly, literally or under the doctrine of equivalents, any claim of the '997 patent;

(b) Declaring that the '997 patent is invalid;

(c) Denying any request by Amgen for injunctive relief;

(d) Finding this case to be exceptional under 35 U.S.C. § 285 and awarding Sandoz its costs and reasonable attorneys' fees;

(e) Awarding Sandoz any other relief as is just and proper.

Dated: February 21, 2019

MORRISON & FOERSTER LLP

By: */s/ Erik J. Olson*
ERIK J. OLSON

Attorneys for Plaintiff
SANDOZ INC.

EXHIBIT A

US009643997B2

(12) **United States Patent**
Shultz et al.

(10) **Patent No.: US 9,643,997 B2**
(45) **Date of Patent: *May 9, 2017**

(54) **CAPTURE PURIFICATION PROCESSES FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM**

(71) Applicant: **AMGEN INC.**, Thousand Oaks, CA (US)

(72) Inventors: **Joseph Edward Shultz**, Binningen (CH); **Roger Hart**, Loveland, CO (US)

(73) Assignee: **AMGEN INC.**, Thousand Oaks, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/599,336**

(22) Filed: **Jan. 16, 2015**

(65) **Prior Publication Data**

US 2015/0361130 A1 Dec. 17, 2015

Related U.S. Application Data

(62) Division of application No. 12/822,990, filed on Jun. 24, 2010, now Pat. No. 8,940,878.

(60) Provisional application No. 61/220,477, filed on Jun. 25, 2009.

(51) **Int. Cl.**

C07K 1/14 (2006.01)
C07K 1/22 (2006.01)
C07K 1/18 (2006.01)
C07K 1/32 (2006.01)
C07K 16/00 (2006.01)

(52) **U.S. Cl.**

CPC ***C07K 1/22*** (2013.01); ***C07K 1/145*** (2013.01); ***C07K 1/18*** (2013.01); ***C07K 1/32*** (2013.01); ***C07K 16/00*** (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,237,224	A	12/1980	Cohen et al.
4,468,464	A	8/1984	Cohen et al.
4,740,470	A	4/1988	Cohen et al.
5,466,377	A	11/1995	Grandics et al.
5,663,304	A	9/1997	Builder et al.
5,922,846	A	7/1999	Cerletti et al.
6,660,843	B1	12/2003	Feige et al.
6,808,902	B1	10/2004	Treuheit et al.
6,972,327	B1	12/2005	Madani et al.
7,138,370	B2	11/2006	Oliner et al.
7,435,804	B2	10/2008	Kordyum et al.
7,442,778	B2	10/2008	Gegg et al.
7,511,012	B2	3/2009	Han et al.
7,723,490	B2	5/2010	Treuheit et al.
8,906,648	B2	12/2014	Butler et al.
8,940,878	B2	1/2015	Shultz et al.
8,952,138	B2	2/2015	Shultz et al.
9,090,684	B2	7/2015	Borras et al.
9,200,030	B2	12/2015	Pizarro et al.
2006/0228329	A1	10/2006	Brady et al.
2007/0238860	A1	10/2007	Schlegl
2008/0214795	A1	9/2008	Ramanan et al.
2008/0260684	A1	10/2008	Dietrich et al.
2010/0267936	A1	10/2010	Treuheit et al.

FOREIGN PATENT DOCUMENTS

DE	10-2005-033250	A1	1/2007
EP	0 657 466	A1	6/1995
EP	1 845 103	A1	10/2007
EP	1845103	A1	5/2015
WO	92/04382	A1	3/1992
WO	97/28272	A1	8/1997
WO	99/42486	A1	8/1999
WO	01/07477	A1	2/2001
WO	02/020762	A3	8/2002
WO	2004/058988	A3	11/2007
WO	2009/023270	A1	2/2009
WO	WO2011/005488	A1	1/2011

OTHER PUBLICATIONS

Cowley & Mackin, "Expression, purification and characterization of recombinant human proinsulin." FEBS Lett 402 124-130 (1997).

Rudolph & Lilie, "In vitro folding of inclusion body proteins," FASEB J. 10 49-56 (1996).

Creighton, T.E., "Renaturation of the reduced bovine pancreatic trypsin inhibitor," J. Mol. Biol. 87:563-577, (1974).

Stöckel, J. et al., "Pathway of detergent-mediated and peptide ligand-mediated refolding of heterodimeric class II major histocompatibility complex (MHC) molecules," Eur J. Biochem 248: 684-691 (1997).

St. John et al., "High pressure refolding of recombinant human growth hormone from insoluble aggregates. Structural transformations, kinetic barriers, and energetics," J. Biol. Chem. 276(50): 46856-63 (2001).

Lilie, Schwarz & Rudolph, "Advances in refolding of proteins produced in *E. coli*." Current Opinion in Biotechnology 9(5): 497-501 (1998).

Tran-Moseman, Schauer & Clark, "Renaturation of *Escherichia coli*-derived recombinant human macrophage colony stimulating factor," Protein Expression and Purification 16(1) 181-189 (1999.

Darby, N.J et al., "Refolding of bovine pancreatic trypsin inhibitor via non-native disulphide intermediates," J. Mol. Biol. 249(2): 463-477, (1995).

Snyder et al , "Characterization of DC-SIGN/R interaction with Human Immunodeficiency Virus Type 1 gp 120 and ICAM Molecules favors the receptor's role as an antigen-capturing rather than an adhesion receptor," J. Virology 79(8):4589-4598, Apr. 2005.

Javaherian, K. et al., "Laminin Modulates Morphogenic Properites of the Collagen XVII Endostatiin Domain," J. Biol. Chem. 277(47):45211-45218, Nov. 22, 2002.

GE Healthcare Instructions 71-7089-00AE: Affinity media. Protein A Sepharose CL-4B. p. 1-8, Mar. 2006 (Cited in JP Office action as D5 http://eclub.biomart.cn/sites/eclub.biomart.cn/themes/aktaclub/Files/71708900AE_UM_Protein_A_Sepharose_CL-4B.pdg, Mar. 2006).

GE Healthcare Instructions 71-5002-60 AE: Ion exchange chromatography; Q Sepharose XL, XL virus licensed. SP Sepharose XL, pp. 1-16, Feb. 2006 (cited in JP Office action as D6https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314723116657/litdoc71500260AE_20110830185637.pdf, Feb. 2006).

(Continued)

Primary Examiner — Brian J Gangle

(74) *Attorney, Agent, or Firm* — Raymond M. Doss

(57) **ABSTRACT**

Methods of purifying proteins expressed in non-mammalian expression systems in a non-native soluble form directly from cell lysate are disclosed. Methods of purifying proteins expressed in non-mammalian expression systems in a non-native limited solubility form directly from a refold solution are also disclosed. Resin regeneration methods are also provided.

30 Claims, 5 Drawing Sheets

(56) **References Cited**

OTHER PUBLICATIONS

Pavlinkova et al., Charge-Modified Single Chain Antibody Constructs of Monoclonal Antibody CC4: Generation, Characterization, Pharmokinetics and Biodistribution Analysis (Nuclear Med. Biol., vol. 26, pp. 27-34, 1999.

Ronnmark et al, "Construction and characterization of affibody-Fe chimeras produced in *Escherichia coli*" (Journal of Immunological Methods, 261:199-211, 2002).

Tengliden, "Development of Cleaning-in-Place Procedures for Protein A Chromatography Resins Using Design of Experiments and High Throughput Screening Technologies", Masters Thesis, Linkoping University, Feb. 2008).

Arvidsson, P et al , "Direct chromatographic capture of enzymer from crude homogenate using immobilized metal affinity chromatography on a continuous supermacroporous adsorbent." Journal of Chromatography 966 (2): 275-290 (2003).

Ling et al., "Integration of mechanical cell disruption and fluidised bed recovery of G3PHD from unclarified disrupted 2 yeast: a comparative study of the performance of unshielded and polymer shielded dye-ligand chromatography systems," J. Biotech. 119(4): 436-448 (2005).

Fischer, B et al , "Isolation renaturation and formation of disulfide bonds of eukaryotic proteins expressed in *Escherichia coli* as inclusion bodies," Biotech. and Bioengineering, 41 (1): 3-13(1993).

Ford et al., "Affinity purification of novel bispecific antibodies recognising carcinoembryonic antigen and doxorubicin," J. Chromatogr. B, 754: 427-435 (2001).

Shukla et al., "Downstream processing of monoclonal antibodies—Application of platform approaches." Journal of Chromatography B, 848(1):28-39 (2007).

Wang et al., "Perturbation of the antigen-binding site and staphylococcal protein A-binding site of IgG before significant changes in global conformation during denaturation: an equillbrium study." Biochem J 325(Part 3) 707-710 (1997).

Hasemann & Capra, "Immunoglobulins: Structure and Function." in William E. Paul, ed., Fundamental Immunology Second Edition, 209, 210-218 (1989).

Ostrove, "Affinity Chromatography: General Methods." Guide to Protein Purification, Methods in Enzymology 182: 371-379 (1990).

Devereux et al., "A comprehensive set of sequence analysis programs for the VAX," Nucleosides, nucleotides & nucleic acids: Nucl. Acids Res. 12: 387-389 (1984).

Gribskov and Burgess, "Sigma factors from *E. coli*, B. subtilis, phage SP01, and phage T4 are homologous proteins." Nucl. Acids Res. 14:6745 (1986).

Gulich, Susanne, et al , "Protein engineering of an IgG-binding domain allows milder elution conditions during affinity chromatography," J. Biol. 76, Issues 2-3, pp. 233-244 (2000).

Ostrove, "Affinity Chromatography; General Methods," Guide to Protein Purification, Methods in Enzymology 182 357-371 (1990).

Stoscheck, C., "Quantitation of Protein." Guide to Protein Purification, Methods in Enzymology 182:50-68 (1990).

Vola et al., "Recombinant proteins Land LG. Two new tools for purification of murine antibody fragments," Cell Biophys. 24-25: 27-36 (1994).

Aybay and Imir, "Development of a rapid, single-step procedure using protein G affinity chromatography to deplete fetal 12 calf serum of its IgG and to isolate murine IgG1 monoclonal antibodies from supernatants of hybridoma cells," Int'l. J Immunol. Methods 23391-2): 77-81 (2000).

Ejima, Daisuke, et al., Effective elution of antibodies by arginine and arginine derivatives in affinity column chromatography. Analytical Biochem. 345250-257 (2005).

Arakawa, Tsutomu et al , "Elution of antibodies from a Protein-A column by acqueous arginine solutions." Protein Express & Purif., 36:244-248 (2004).

Miller, Timothy et al., The rapid isolation of ribonuclease-free immunoglobulin G by protein A-sepharose affinity chromatography, J. Immun. Methods 24: 111-125 (1978).

Singh et al., "Solubilation and Refolding of Bacteria Inclusion Body Proteins." J. Bioscience and Bioengineering. vol. 99(4), pp. 3o3-310 (2005).

Gottschalk, U. "Filtration and Purification in the Biopharmaceutical Industry," ebook CRC Press 2007, eds. Maik W. Jornitz and Theodore H. Meltzer, 2nd ed, pp. 459-495.

US District Court, Southern District of Florida; Case No. 15-61631-CIV-COHN (consolidated with 15-62081-CIV-COHN) *Amgen Inc. and Amgen Manufacturing Limited* v. *Apotex Inc. and Apotex Corp*; Defendants Apotex Inc. and Apotex Corp.; Invalidity Contentions; Dec. 1, 2015.

US District Court, Southern District of Florida; Case No. 15-cv-61631-JIC/BSS; *Amgen Inc. and Amgen Manufacturing Limited* v. *Apotex Inc. and Apotex Corp*; Defendants Apotex Inc. and Apotex Corp.; "Pegfil—Invalidity Contentions" Oct. 19, 2015.

Appendix A: Prior Art Chart for U.S. Pat. No. 8,952,138; Pegfil Invalidity Claim Chart 2015.

DeBernadez Clark, E., "Refolding of recombinant proteins," *Current Opinion in Biotechnology*, v:9, pp. 157-163 (1998).

DeBernadez Clark, E., "Protein Refolding for industrial processes," *Current Opinion in Biotechnology*, v12 i:2, pp. 202-207 (2001).

DeBernadez Clark, E., Oxidative Renaturation of Hen Egg-White Lysozyme, Folding Aggregation, *Biotechnology Progress*, v:14 i:1, pp. 47-54 (1998).

UniProtKB—Q6EBC2 (IL31_HUMAN) UniProt (2016), pp. 1-8 http://www.uniprot.org/uniprot/Q6EBC2.

UniProtKB—Q6EAL8 (IL31_MOUSE) UniProt (2016), pp. 1-7.

IUPAC Gold Book—dalton (2016). *Amgen Exhibit 2014 : Apotex Inc. et al.* V. *Amgen Inc. et al.*, (IPR2016-01542), pp. 1.

Protein Data Bank, Hen Egg White Lysozyme, http://www.rcsb.org/pdb/explore/explore.do?structureId=193L; http://www.rcsb.org/pdb/explore/remediatedSequence.do?structureId=193L, 2016.

DeBernadez Clark, Eliana, *Refolding of recombinant proteins*, Current Opinion in Biotechnology, vol. 9, pp. 157-163 (1998).

Kamau, Samuel M. et al. (2010). Alpha-Lactalbumin: Its Production Technologies and Bioactive Peptides. *Comprehensive Reviews in Food Science and Food Safety*, pp. 197-212, vol. (9).

Lehninger, Albert L. (1982). Chapter 17: Electron Transport, Oxidative Phosphorylation, and Regulation of ATP production. *Principles of Biochemistry*. New York, New York: Worth Publishers, Inc., pp. 1-46.

Lu et al. (May 5, 1992). Folding and Oxidation of Recombinant Human Granulocyte Colony Stimulating Factor Produced in *Escherichia coli*: Characterization of the disulfide-reduced intermediates and cysteine→Serine Analogs. *The Journal of Biological Chemistry*, pp. 8770-8777.

Phillips, David C. (1966). The Three-dimensional Structure of an Enzyme Molecule. Scientific American, pp. 78-90.

Schrodel and De Marco (May 31, 2015). Characterization of the aggregates formed during recombinant protein expression in bacteria. *BMC Biochemistry*. DOI: 10.1186/1471-2091-6-10, pp. 1-11.

Slanger, Charles J. et al. (1999). Use of Mass Spectrometry to Rapidly Characterize the Heterogeneity of Bovine α-Lactalbumin. *Department of Product Technology, BIZO Food Research*. vol. (47), pp. 4549-4556.

Yamaguchi, Satoshi, et al. (2013). Protein refolding using chemical refolding additives. *Biotechnology Journal*, v (8), pp. 17-31.

United States Patent and Trademark Office Before the Patent Trial and Appeals Board. (Nov. 23, 2016). Patent Owners' Preliminary Response: *Apotex Inc. and Apotex Corp. Petitioners* v. *Amgen Inc. and Amgen Manufacturing Limited, Patent Owners* (Case IPR2016-01542, U.S. Pat. No. 8,952,138), pp. i-v, pp. 1-51.

United States Patent and Trademark Office Before the Patent Trial and Appeals Board. (Aug. 29, 2016). Patent Owner's Mandatory Notices: *Apotex Inc. and Apotex Corp. Petitioners* v. *Amgen Inc. and Amgen Manufacturing Limited, Patent Owners* (Case IPR2016-01542, U.S. Pat. No. 8,952,138), pp. 1-3.

United States Patent and Trademark Office Before the Patent Trial and Appeals Board. (Aug. 25, 2016). Notice of Filing Date Accorded to Petition and Time for Filing Patent owner Preliminary Response: *Apotex Inc. and Apotex Corp. Petitioner* v. *Amgen Inc. and Amgen Manufacturing Limited, Patent Owner* (Case IPR2016-01542, U.S. Pat. No. 8,952,138), pp. 1-5.

(56) **References Cited**

OTHER PUBLICATIONS

United States Patent and Trademark Office Before the Patent Trial and Appeals Board. (Aug. 25, 2016). Petition for Inter Partes Review of U.S. Pat. No. 8,952,138 Under 35 U.S.C. §§ 311-319 and 37 C.F.R. §§ 42.1-.80, 42.100-.123: *Apotex Inc. and Apotex Corp. Petitioners* v. *Amgen Inc. and Amgen Manufacturing Limited, Patent Owner* (Inter Partes Review No. IPR2016-01542), pp. i-viii, pp. 1-69.
United States Patent and Trademark Office Before the Patent Trial and Appeals Board. (Aug. 29, 2016). Updated Mandatory Notices for Patent Owners: *Apotex Inc. and Apotex Corp. Petitioners* v. *Amgen Inc. and Amgen Manufacturing Limited, Patent Owners* (Case IPR2016-01542, U.S. Pat. No. 8,952,138), pp. 1-4.
United States Patent and Trademark Office Before the Patent Trial and Appeal Board. (Nov. 23, 2016). Declaration, pp. 1-81, Curriculum Vitae of Richard C. Willson, PhD.pp. 1-7: *Apotex Inc. and Apotex Corp.* v. *Amgen Inc. and Amgen Manufacturing Limited* (Inter Partes Review No. IPR2016-01542, U.S. Pat. No. 8,952,138).
United States Patent and Trademark Office Before the Patent Trial and Appeal Board. (Aug. 5, 2016). Declaration pp. 1-74, Curriculum Vitae of Anne S. Robinson, PhD. pp. 1-7, Appendix A, pp. 1-4: *Apotex Inc. and Apotex Corp.* v. *Amgen Inc. and Amgen Manufacturing Limited* (Inter Partes Review No. IPR2016-01542).
United States Patent and Trademark Office. *Notice of Allowance and Fees Due*, U.S. Appl. No. 12/820,087, pp. 1-3.
United States Patent and Trademark Office. Yunsoo Kim, Examiner. (Jan. 9, 2012). Information Disclosure Statement by Applicant, pp. 1-2.
Unites States District Court Southern District of Florida. (Apr. 7, 2016). Claim Construction Order, Document 119: *Amgen Inc. and Amgen Manufacturing Limited, Plaintiffs*, v. *Apotex Inc. and Apotex Corp., Defendants* (Case No. 15-61631-CIV-COHN/SELTZER), pp. 1-12.
Unites States District Court Southern District of Florida. (Sep. 6, 2016), Findings of Fact and Conclusions of Law, Document 267: *Amgen Inc. and Amgen Manufacturing Limited, Plaintiffs*, v. *Apotex Inc. and Apotex Corp., Defendants* (Case No. 15-61631-CIV-COHN/SELTZER, Consolidated with 15-62081-CIV-COHN/SELTZER), pp. 1-30.
Unites States District Court for the Southern District of Florida. (Jul. 18, 2016). Partial Findings Regarding Apotexs Assertion of Invalidity of the 138 Patent. Signed by Judge James I. Cohn. Document 245: *Amgen Inc. and Amgen Manufacturing Limited, Plaintiff*, v. *Apotex Inc. and Apotex Corp., Defendants* (Case No. 15-cv-61631-JIC), pp. 1-5..
Hevehan and Clark, "Oxidative Renaturation of Lysozyme at High Concentrations," Biotechnology and Bioengineering, 1996, 54(3): 221-230.
Hakim and Benhar, "Inclonals," mAbs, published online May 1, 2009, 1:3, 281-287.
Whitford, "Proteins: Structure and Function," Sep. 1, 2005.
Johnson, "Human insulin from recombinant DNA technology". Science (1983) 219 (4585): 632-637.
Vallejo et al. "Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins," Microbial Cell Factories (2004) 3, 1-12.
Neubauer et al. "Protein inclusion bodies in recombinant bacteria. Inclusions in Prokaryotes." Microbiology Monographs Edited by: Shively JM. Springer; (2006) 237-292.
Georgiou and Valax, "Isolating Inclusion Bodies from Bacteria", Chapter 3 in Methods in Enzymology, vol. 309, p. 48-58 (1999) Academic Press.
Palmer and Wingfield "Preparation and Extraction of Insoluble (Inclusion-Body) Proteins from *Escherichia coli*" Curr Protoc Protein Sci. (2004) Nov.; Chapter: Unit-6.3. doi:10.1002/0471140864.ps0603s38.
Shortle et al., "Clustering of Low-Energy Conformations Near the Native Structures of Small Proteins," Proc Natl Acad Sci (1998) 95, 11158-62.
Panda, "Bioprocessing of Therapeutic Proteins from the Inclusion Bodies of *Escherichia coli*" Adv Biochem Engin/Biotechnol (2003) 85: 43-93.
Vincentelli, "High-throughput automated refolding screening of inclusion bodies," Protein Science (2004) 13:2782-2792.
Willis et al., "Investigation of protein refolding using a fractional factorial screen: A study of reagent effects and interactions." Protein Science (2005) 14(7), 1818-1826.
Jungbauer and Kaar "Current status of technical protein refolding," Journal of Biotechnology 128 (2007) 587-596.
Ferrer-Miralles et al. "Microbial factories for recombinant pharmaceuticals" Microbial Cell Factories (2009) 8:17.
Graumann and Premsaller, "Manufacturing of recombinant therapeutic proteins in microbial systems," Biotech J. (2006) 1:164-186.
Xie and Wetlaufer, "Control of aggregation in protein refolding: The temperature-leap tactic," Protein Science (1996) 5:517-523.
Puri, "Refolding of recombinant porcine growth hormone in a reducing environment limits in vitro aggregate formation," FEBS (1991) vol. 292, No. 1.2, 187-190.
Ejima, "High yield refolding and purification process for recombinant human interleukin-6 expressed in *Escherichia coli*," Biotechnology and Bioengineering (1999) vol. 62, No. 3, 301-310.
Patra et al., "Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coli*," Protein Expression and Purification (2000) 18, 182-192.
Mannall et al., "Factors Affecting Protein Refolding Yields in a Fed-Batch and Batch-Refolding System," Biotechnology and Bioengineering, (2007) vol. 97, No. 6, 1523-1534.
Misawa and Kumagai "Refolding of Therapeutic Proteins Produced in *Escherichia coli* as Inclusion Bodies" Biopoly (1999) 51: 297-307.
Park et al., "A Divalent Recombinant Immunotoxin Formed by a Disulfide Bond between the Extension Peptide Chains," Mol. Cells (2001) vol. 12, No. 3, 398-402.
EnbrelTM (etanercept) label, Nov. 1998.
Bolado, "Amgen Opens Trial in Fight Over Neulasta Generic," Law360, Jul. 11, 2016 (http://www.law360.com/articles/814748/amgen-opens-trial-in-fight-over-neulasta-generic).
Maurer et al., "Folding and aggregation of a multi-domain engineered immunotoxin," Biochemical Engineering Journal (2013) 81:8-14.
Sereikaite et al., "Production of recombinant mink growth hormone in *E. coli*," Appl Microbiol Biotechnol (2007) 74:316-323.
Shimamoto et al., "Peptibodies: A flexible alternative format to antibodies," mAbs (Sep./Oct. 2012) 4:5, 586-591.
Bowden et al., "Structure and morphology of protein inclusion bodies in *Escherichia coli*" Bio/Tech (1991) 9:725-730.
Weiss et al. "Principles, Approaches, and Challenges for Predicting Protein Aggregation Rates and Shelf Life" J Pharm Sci. Apr. 2009; 98(4):1246-77.
Ventura and Villaverde "Protein quality in bacterial inclusion bodies" Trends in Biotechnology vol. 24 No. 4 Apr. 2006.
United States Patent and Trademark Office Before the Patent Trial and Appeal Board. (Feb. 17, 2017). Decision Granting Institution of Inter Partes Review 37 C.F.R. § 42.108: *Apotex Inc. and Apotex Corp. Petitioner* v. *Amgen Inc. and Amgen Manufacturing Limited, Patent Owner* (Case IPR2016-01542, U.S. Pat. No. 8,952,138 B2), pp. 1-35.
United States District Court Document for the Southern District of Florida. (Dec. 11, 2015). Document 77: *Amgen Inc. and Amgen Manufacturing Limited, Plaintiffs*, v. *Apotex Inc. and Apotex Corp., Defendants* (Case No. 15-cv-61631-JIC/BSS), pp. 1-23.
United States District Court Document for the Southern District of Florida. (Dec. 11, 2015). Document 77-4: Exhibit 4, *Amgen Inc. and Amgen Manufacturing Limited, Plaintiffs*, v. *Apotex Inc. and Apotex Corp., Defendants* (Case No. 15-cv-61631-JIC/BSS), pp. 1-16.
United States District Court Document for the Southern District of Florida. (Jan. 8, 2016). Document 83-1: Exhibit 1, *Amgen Inc. and Amgen Manufacturing Limited, Plaintiffs*, v. *Apotex Inc. and Apotex Corp., Defendants* (Case No. 15-cv-61631-JIC/BSS), pp. 1-19.

(56) **References Cited**

OTHER PUBLICATIONS

Document 35: Answer and Affirmative Defenses to Complaint, Counterclaim against All Plaintiffs by Apotex Corp., Apotex Inc. (Brier, Simeon) (Entered: Oct. 5, 2015), pp. 1-41.
Document 42: Plaintiffs Motion for Preliminary Injunction and Incorporated Memorandum of Law by Amgen Inc., Amgen Manufacturing Limited, pp. 1-25. Attachments: #1 Affidavit Declaration of Robert Azelby, pp. 1-4. # 2, Affidavit Declaration of Nicholas Groombridge, pp. 1-4. # 3 Exhibit A—Apr. 17, 2015 Letter to Groombridge—Pegfilgrastim, pp. 1-2. # 4 Exhibit B—May 8, 2015 Ltr. Groombridge to Coblentz re pegfilgrastim (1)(8)(A) notice, pp. 1-3. # 5 Exhibit C—Jul. 29, 2015 N Groombridge letter to B Coblentz, pp. 1-3. # 6 Exhibit D—Aug. 24, 2015 Letter to Groombridge, pp. 1-2. # 7 Exhibit E—May 5, 2015 Order Granting Motion for an Injunction Pending Appeal, pp. 1-3. # 8 Exhibit F—Joint Stip Re Amgens Motion for Preliminary Injunction Jan. 10, 2015, pp. 1-5. # 9 Exhibit G—FDA Website Printout, pp. 1-3. # 10 Exhibit H—Jorge Mestre-Ferrandiz, et al., The R&D Cost of a New Medicine (2012), pp. 1-101. # 11 Exhibit I—Oct. 16, 2015 Order Denying Petition for En Banc Rehearing, pp. 1-3. # 12 Text of Proposed Order Proposed Order)(O'Sullivan, John) (Entered: Oct. 16, 2015), pp. 1-2.
Document 47: Apotex's Corrected Answer, Affirmative Defenses & Counterclaims to Amgen's Complaint, Oct. 23, 2015, pp. 1-42.
Document 64: Defendants Apotex's Answer, Affirmative Defenses and Counterclaims to Plaintiffs' Complaint, Dec. 1, 2015, pp. 1-45.
Document 76: Apotex's Opening Claim Construction Brief, Dec. 11, 2015, pp. 1-26.
Document 77: Amgen's Opening Claims Construction Brief, Dec. 11, 2015, pp. 1-23; Document 77-1 to Exhibit 1, U.S. Pat. No. 8,952,138, document entered on Dec. 11, 2015, pp. 1-19; Document 77-2 to Exhibit 2, U.S. Pat. No. 6,162,427, document entered Dec. 11, 2015, pp. 1-7; Document 77-3 to Exhibit 3, U.S. Pat. No. 5,824,784, document entered Dec. 11, 2015, pp. 1-31; Document 77-4 to Exhibit 4, Declaration of Richard C. Willson, Ph. D. Regarding Claim Constructions of Shultz et al., document entered Dec. 11, 2015, pp. 1-16, Document 77-5 to Exhibit A, Richard Willson CV, document entered Dec. 11, 2015, pp. 1-21; Document 77-6 to Exhibit B, Effective renaturation of denatured and reduced immunoglobulin G in vitro without assistance of chaperone, document entered on Dec. 11, 2015, pp. 1-7; Document 77-7 to Exhibit C, Effective renaturation of reduced lysosome by gentle removal of urea, document entered on Dec. 11, 2015, pp. 1-6; Document 77-8 to Exhibit D, Perspectives in Biochemistry, *Biochemistry*, document entered on Dec. 11, 2015, pp. 1-10; Document 77-9 to Exhibit E, Structural Stability of Covalently Linked GroES Heptamer: Advantages in the Formulation of Oligomeric Structure, *Science Direct*, document entered on Dec. 11, 2015, pp. 1-16.
Document 82: Brief in response to Plaintiffs' Opening Claim Construction Brief by Apotex Corp., Apotex Inc. re 77 Trial Brief, documents entered on Jan. 8, 2016, pp. 1-25. Attachments: # 1 Declaration of W. Blake Coblentz in Support of Defendants Responsive Claim Construction Brief, pp. 1-2. # 2 Exhibit A—Declaration of Anne S. Robinson, Ph.D., pp. 1-17. # 3 Exhibit B—R. Rudolph and H. Lilie, In vitro folding of inclusion body proteins). Entered: Jan. 8, 2016, pp. 1-9.
Document 83: Responsive Claim Construction Brief by Amgen Inc., Amgen Manufacturing Limited. Documents entered on Jan. 8, 2016. pp. 1-25. Attachments: # 1 Affidavit Exh. 1—Rebuttal Declaration of Richard C. Willson, pp. 1-19. # 2 Exhibit A—Feb. 23, 2012 Office Action Response, pp. 1-6. # 3 Exhibit B—'370 Patent, pp. 1-167. # 4 Exhibit C—Jan. 29, 2014 Office Action, pp. 1-8. # 5 Exhibit D—Apr. 28, 2014 Office Action Response, # 6 Affidavit Exh. 2—Rebuttal Declaration of Louis M. Pelus, pp. 1-22. # 7 Exhibit A—Pelus CV, pp. 1-49. # 8 Exhibit B—Richman, pp. 1-11. # 9 Exhibit C—Shirota, pp. 1-8. # 10 Exhibit D—Neben, pp. 1-10.
Document 89: Apotex's Reply to Plaintiffs' Responsive Claim Construction Brief. Notice by Apotex Corp., Apotex Inc. re 83 Trial Brief,, Defendants Apotex Inc. and Apotex Corp.'s Reply to Plantiffs' Responsive Claim Construction Brief. Documents entered on Jan. 27, 2016, pp. 1-16. Attachments: # 1 Declaration of W. Blake Coblentz in Support of Defendants' Reply to Plaintiffs' Responsive Claim Construction Brief, pp. 1-2 # 2 Exhibit A—Excerpts of deposition transcript of Richard C. Willson, III, Ph.D., pp. 1-47 # '3 Exhibit B—Excerpts of deposition transcript of Anne Robinson, Ph.D., pp. 1-5 # 4 Exhibit C—Excerpts of deposition transcript of Louis M. Pelus, Ph.D. pp. 1-16.
Document 90: Amgen's Reply Claim Construction Brief. Document entered on Jan. 27, 2016, pp. 1-15. Attachments: Exhibit 1; Videotaped Deposition of Richard C. Willson, Ph. D. Jan. 18, 2016; pp. 1-109 Exhibit 2: Videotaped Deposition of Louis M. Pelus, Ph.D. Jan. 19, 2016; pp. 1-33. Exhibit 3: Transcript of the Testimony of Videotaped Deposition of Anne Robinson, Ph. D., Jan. 20, 2016; pp. 1-60. Exhibit 4: Videotaped Deposition of David T. Scadden, M.D. Jan. 22, 2016; pp. 1-43.
Document 184: Brief Plaintiffs Supplemental Claim Construction Brief Regarding the Meaning of "Protein" in Claim 1 of the '138 Patent by Amgen Inc., Amgen Manufacturing Limited. Documents Entered: Jun. 22, 2016), pp. 1-15. Attachments: #1 Exhibit Ex. 1—U.S. Pat. No. 8,952,138, pp. 1-19. # 2 Exhibit Ex. 5—Jan. 18, 2016 Willson Dep. Tr. (Excerpt), pp. 1-12. # 3 Exhibit Ex. 6—Jun. 22, 2012 Response to Office Action from File History of U.S. Pat. No. 8,952,138)(O'Sullivan, John), pp. 1-6.
Document 186: Trial Brief Apotex's Supplemental Claim Construction Brief in Support of Their Construction for the Term 'protein' As Used in Claim 1 of U.S. Pat. No. 8,952,138 by Apotex Corp., Apotex Inc.pp. 1-18 (Attachments: #1 Exhibit 1 U.S. Pat. No. 8,952,138; pp. 1-19; # 2 Exhibit 6—Response to Office Action dated Jun. 22, 2012)(Brier, Simeon) (Entered: Jun. 22, 2016); pp. 1-6.
Document 244: Motion for Judgment on Partial Findings Pursuant to Fed. R. Civ. P. 52(c) by Apotex Corp., Apotex Inc. pp. 1-12, Entered: Jul. 18, 2016: Attachments #1 Exhibit A—Pages from Trial Transcript Day 1 (Jul. 11, 2016) (Willson), pp. 1-6 # 2 Exhibit B—Pages from Trial Transcript Day 2 (Jul. 12, 2016) (Willson), pp. 1-9 # 3 Exhibit C—Pages from Trial Transcript Day 3 (Jul. 13, 2016) (Dowd), pp. 1-13 # 4 Text of Proposed Order, pp. 1.
Document 247:Transcript of Bench Trial held on Jul. 11, 2016 before Judge James I. Cohn, 1-245 pages, Court Reporter: Karl Shires, 954-769-5496 / Karl_Shires@flsd.uscourts.gov. Transcript may be viewed at the court public terminal or purchased by contacting the Court Reporter/Transcriber before the deadline for Release of Transcript Restriction. After that date it may be obtained through PACER. Redaction Request due Aug. 11, 2016. Redacted Transcript Deadline set for Aug. 22, 2016. Release of Transcript Restriction set for Oct. 20, 2016. (Shires, Karl) (Entered: Jul. 18, 2016).
Document 248: Transcript of Bench Trial held on Jul. 12, 2016 before Judge James I. Cohn, 1-171 pages, Court Reporter: Karl Shires, 954-769-5496 / Karl_Shires@flsd.uscourts.gov. Transcript may be viewed at the court public terminal or purchased by contacting the Court Reporter/Transcriber before the deadline for Release of Transcript Restriction. After that date it may be obtained through PACER. Redaction Request due Aug. 11, 2016. Redacted Transcript Deadline set for Aug. 22, 2016. Release of Transcript Restriction set for Oct. 20, 2016. (Shires, Karl) (Entered: Jul. 18, 2016).
Document 249: Transcript of Bench Trial held on Jul. 13, 2016 before Judge James I. Cohn, 1-61 pages, Court Reporter: Karl Shires, 954-769-5496 / Karl_Shires@flsd.uscourts.gov. Transcript may be viewed at the court public terminal or purchased by contacting the Court Reporter/Transcriber before the deadline for Release of Transcript Restriction. After that date it may be obtained through PACER. Redaction Request due Aug. 11, 2016. Redacted Transcript Deadline set for Aug. 22, 2016. Release of Transcript Restriction set for Oct. 20, 2016. (Shires, Karl) (Entered: Jul. 18, 2016).
Document 250: Transcript of Bench Trial held on Jul. 14, 2016 before Judge James I. Cohn, 1-242 pages, Court Reporter: Karl Shires, 954-769-5496 / Karl_Shires@flsd.uscourts.gov. Transcript may be viewed at the court public terminal or purchased by contacting the Court Reporter/Transcriber before the deadline for Release of Transcript Restriction. After that date it may be obtained through PACER. Redaction Request due Aug. 11, 2016. Redacted

(56) **References Cited**

OTHER PUBLICATIONS

Transcript Deadline set for Aug. 22, 2016. Release of Transcript Restriction set for Oct. 20, 2016. (Shires, Karl) (Entered: Jul. 18, 2016).
Document 254: Response in Opposition re 244 Motion for Judgment on Partial Findings Pursuant to Fed. R. Civ. P. 52(c) filed by Amgen Inc., Amgen Manufacturing Limited. Replies due by Aug. 15, 2016; pp. 1-20.
Document 259: Mandate of US Federal Circuit (certified copy) Affirm Judgment/ Order of the district court with courts opinion re 72 Notice of Appeal, filed by Apotex Corp., Apotex Inc. ; Date Issued: Aug. 11, 2016 ; US Federal Circuit Case Number: 16-1308 (amb) (Entered: Aug. 11, 2016); pp. 1-26.
Document 260: Reply to Response to Motion re 244 Motion for Judgment on Partial Findings Pursuant to Fed. R. Civ. P. 52(c) filed by Apotex Corp., Apotex Inc. pp. 1-11; Attachments: # 1 Exhibit 1—Trial Transcript Day 2 (Jul. 12, 2016) (Willson), pp. 1-7. # 2 Exhibit 2—Trial Transcript Day 3 (Jul. 13, 2016) (Dowd), pp. 1-4. # 3 Exhibit 3—Trial Transcript Day 4 (Jul. 14, 2016) (Robinson), pp. 1-4. # 4 Exhibit 4—Trial Transcript Day 5 (Jul. 18, 2016), pp. 1-3.
Document 268: Final Judgment Signed by Judge James I. Cohn on Sep. 6, 2016. (tpl) Notice: If there are sealed documents in this case, they may be unsealed after 1 year or as directed by Court Order, unless they have been designated to be permanently sealed. See Local Rule 5.4 and Administrative Order 2014-69. (Entered: Sep. 6, 2016); pp. 1-5.

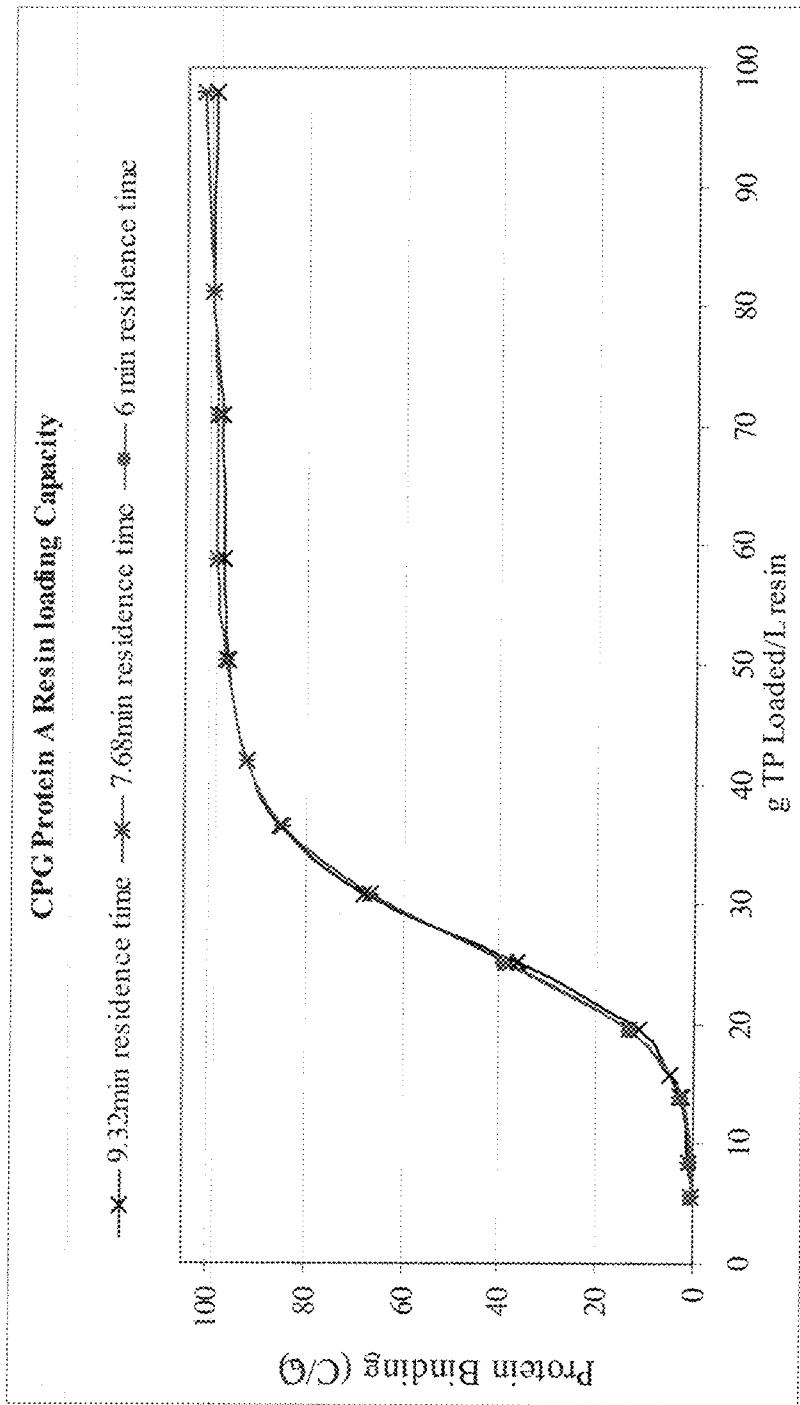

Figure 1

		Average Purity					
		RP-HPLC Main Peak Purity (%)	SE-HPLC Main Peak Purity (%)	CE-SDS Main Peak Purity (%)	Host Protein Level (ppm)	DNA Level (pg/mg protein)	Average Yield (%)
Load	Average (n=13)	34.5	74.5	79.2	9100.0	>70000	-
	Std. Dev (n=13)	2.4	2.7	4.4	424.3	*	-
Purified Pool	Average (n=17)	41.3	68.8	84.7	41.0	215.2	81.7
	Std. Dev (n=17)	1.5	3.8	4.0	5.7	301.2	12.3

* Data limited to N=1

Figure 2

		Average Purity					
		RP-HPLC Main Peak Purity (%)	SE-HPLC Main Peak Purity (%)	CE-SDS Main Peak Purity (%)	Host Protein Level (ppm)	DNA Level (pg/mg protein)	Average Yield (%)
Load	Average (n=5)	36.0	76.1	75.5	1400.0	>70000	-
	Std. Dev (n=5)	0.9	1.9	1.5	*	*	-
Purified Pool	Average (150 cycles)	40.2	75.0	82.4	71.4	89.2	84.3
	Std. Dev (150 cycles)	2.5	8.7	4.6	23.0	175.0	18.8

* Data limited to N=1

Figure 3

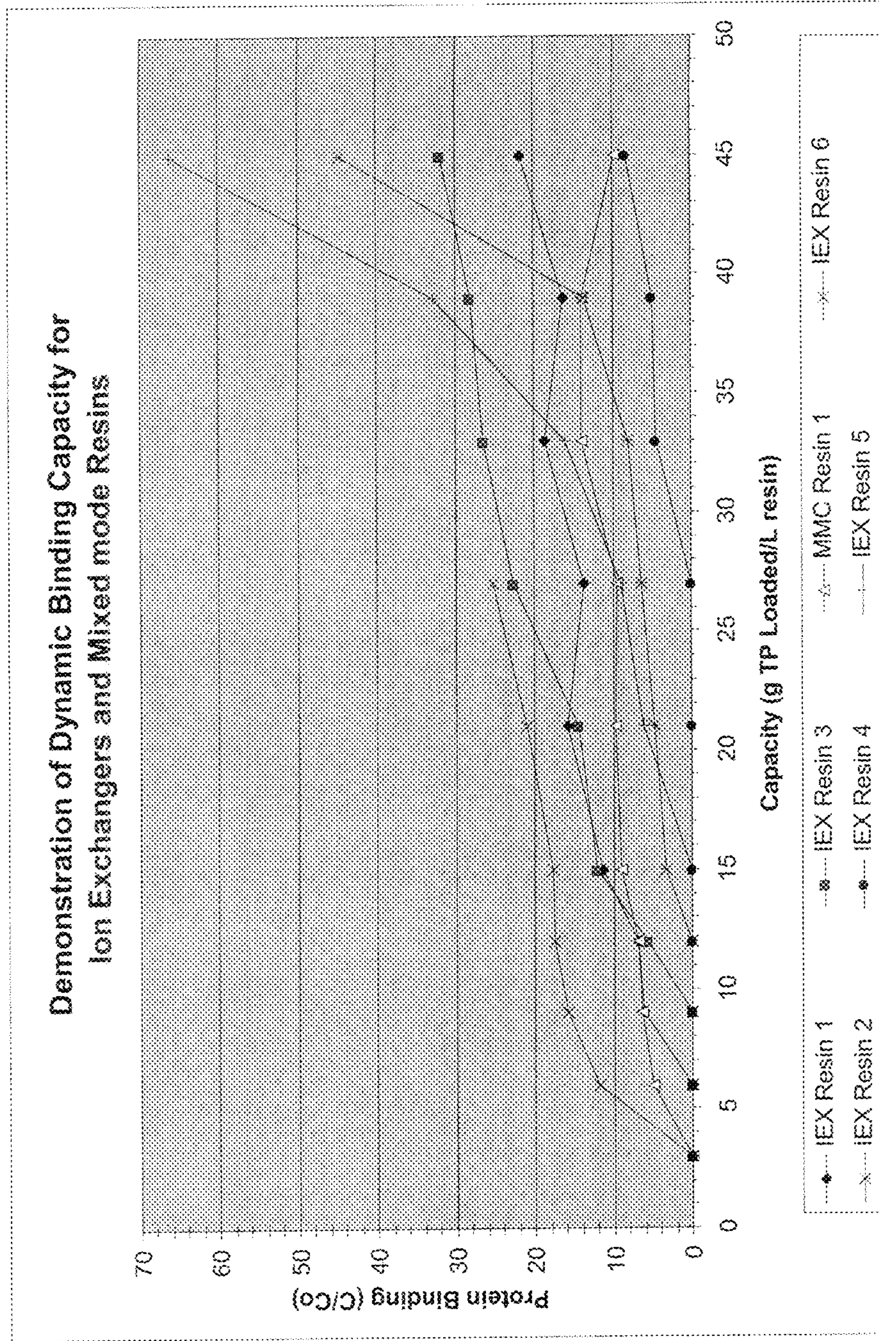

Figure 4

	RP-HPLC Main Peak Purity (%)	SE-HPLC Main Peak Purity (%)	Average Yield (%)
Load	29.8	64.6	-
CEX	46.0	80.3	62.0
AEX	30.9	75.7	85.0

Figure 5

CAPTURE PURIFICATION PROCESSES FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM

This application is a divisional of U.S. application Ser. No. 12/822,990, filed on Jun. 24, 2010, now U.S. Pat. No. 8,940,878; which claims the benefit of U.S. Provisional Application No. 61/220,477 filed Jun. 25, 2009, which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates generally to processes for purifying proteins expressed in non-mammalian systems in both non-native soluble and non-native insoluble forms, and more particularly to the direct capture of such proteins from a refold mixture or a cell lysate pool by a separation matrix.

BACKGROUND OF THE INVENTION

Fc-containing proteins are typically expressed in mammalian cells, such as CHO cells. The use of affinity chromatography to purify Fc-containing proteins is documented (see, e.g., Shukla et al., (2007) *Journal of Chromatography B* 848(1):28-39) and is successful, in part, due to the degree of Fc structure observed in proteins expressed in such systems. Fc-containing proteins expressed in non-mammalian cells, however, are often deposited in the expressing cells in limited solubility forms, such as inclusion bodies, that require refolding, and this has been a limiting factor in selecting non-mammalian systems for expressing Fc-containing proteins.

A drawback to the use of Protein A, Protein G and other chemistries is that in order for a protein comprising an Fc region to associate with the Protein A or Protein G molecule, the protein needs to have a minimum amount of structure. Often, the requisite amount of structure is absent from proteins expressed recombinantly in a soluble, but non-native, form and consequently Protein A chromatography is not performed in a purification process.

In the case of a protein expressed in an insoluble non-native form, Protein A chromatography is typically not performed in a purification process until after the protein has been refolded to a degree that it can associate with the Protein A molecule and has been subsequently diluted out of its refold solution. This is because it was believed that after a protein has been refolded it was necessary to dilute or remove the components of the refold mixture in a wash step, due to the tendency of the components that typically make up a refold solution to disrupt interactions between the target protein and the Protein A molecules (Wang et al., (1997). *Biochem. J.* 325(Part 3):707-710). This dilution step can consume time and resources which, when working at a manufacturing scale of thousands of liters of culture, can be costly.

The present disclosure addresses these issues by providing simplified methods of purifying proteins comprising Fc regions that are expressed in non-mammalian expression systems in a non-native soluble form or in a non-native insoluble form.

SUMMARY OF THE INVENTION

A method of purifying a protein expressed in a non-native soluble form in a non-mammalian expression system is provided. In one embodiment the method comprises (a) lysing a non-mammalian cell in which the protein is

expressed in a non-native soluble form to generate a cell lysate; (b) contacting the cell lysate with an separation matrix under conditions suitable for the protein to associate with the separation matrix; (c) washing the separation matrix; and (d) eluting the protein from the separation matrix.

The protein can be a complex protein, such as a protein is selected from the group consisting of a multimeric protein, an antibody and an Fc fusion protein. The non-mammalian expression system can comprise bacteria or yeast cells. The separation matrix can be an affinity resin, such as an affinity resin selected from the group consisting of Protein A, Protein G and a synthetic mimetic affinity resin, or it can be a non-affinity resin, such as a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin. The cell lysate can be filtered before it is contacted with the separation matrix. Although not required, the method can further comprise refolding the protein to its native form after it is eluted from the separation matrix.

A method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system is provided. In one embodiment that method comprises (a) expressing a protein in a non-native limited solubility form in a non-mammalian cell; (b) lysing a non-mammalian cell; (c) solubilizing the expressed protein in a solubilization solution comprising one or more of the following: (i) a denaturant; (ii) a reductant; and (iii) a surfactant; (d) forming a refold solution comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of the following: (i) a denaturant; (ii) an aggregation suppressor; (iii) a protein stabilizer; and (iv) a redox component; (e) applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix; (f) washing the separation matrix; and (g) eluting the protein from the separation matrix.

The non-native limited solubility form can be a component of an inclusion body. The protein can be a complex protein, such as a complex protein selected from the group consisting of a multimeric protein, an antibody, a peptibody, and an Fc fusion protein. The non-mammalian expression system can be bacteria or yeast cells. The denaturant can comprise one or more of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea, the reductant can comprise one or more of cysteine, DTT, beta-mercaptoethanol and glutathione, the surfactant can comprise one or more of sarcosyl and sodium dodecylsulfate, the aggregation suppressor can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes, the protein stabilizer can comprise one or more of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes, and the redox component can comprise one or more of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol. The separation matrix can be an affinity resin such as an affinity resin selected from the group consisting of Protein A, Protein G, and synthetic mimetic affinity resin or the separation matrix can be a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

In other embodiments, the disclosed methods can further comprise the steps of (a) washing the separation matrix with

a regeneration reagent; and (b) regenerating the separation matrix. The regeneration reagent can be one of a strong base, such as sodium hydroxide or a strong acid, such as phosphoric acid. The regenerating can comprise washing the separation matrix with a solution comprising one or both of a chaotrope present at a concentration of 4-6 M and a reductant. The chaotrope can be one of urea, dimethyl urea, methylurea, ethylurea, and guanidinium, and the reductant can be one of cysteine, DTT, beta-mercaptoethanol and glutathione. In a particular embodiment the regenerating comprises washing the separation matrix with a solution comprising 50 mM Tris, 10 mM citrate, 6M urea, 50 mM DTT at pH 7.4.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a plot demonstrating the binding of refolded, non-mammalian non-native limited solubility fraction complex protein, to Protein A media; in the figure the X denotes resin loading at a 9.32 min residence time, star denotes resin loading at a 7.68 min residence time and solid circles denote resin loading at a 6 min residence time.

FIG. **2** is a table demonstrating purification of a complex protein comprising an Fc domain using Protein A resin.

FIG. **3** is a table demonstrating the reusability of Protein A resin when used to capture a non-mammalian non-native limited solubility complex protein over 150 cycles using the disclosed methods.

FIG. **4** is a plot demonstrating the binding profiles of a refolded, non-mammalian non-native limited solubility complex protein to six different ion exchange resins (IEX Resins 1, 2, 3, 4, 5, 6, corresponding to Toyopearl SP550C™, Toyopearl SP650M™, GigaCAP S™, POROS HS50™, Toyopearl SP650C™ and GE Healthcare SPxL™, respectively) and a mixed-mode resin (MMC Resin 1, GE Healthcare MMC™) following capture using the disclosed methods.

FIG. **5** is a table demonstrating purification levels achieved for a protein comprising an Fc domain using one anion exchange resin (Fractogel TMAE™) and one cation exchange resin (Fractogel SO_3^-™).

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure provides methods of capturing on a separation matrix non-native proteins produced in microbial cells. In the case of the direct capture of a protein expressed in a non-native soluble form the advantages of the present invention over typical processes include enhanced protein concentration, volume reduction, and increased recovery over traditional methods, improved protein stability, and ultimately process cost savings.

In the case of the direct capture of a protein expressed in a non-native limited solubility form, the advantages of the present invention over typical processes include the elimination of the need to dilute the protein out of a refold solution prior to capturing it on a separation matrix.

Another advantage of the disclosed methods is that they may be performed at a range of scales, from laboratory scale (typically milliliter or liter scale), a pilot plant scale (typically hundreds of liters) or on an industrial scale (typically thousands of liters). The application of the disclosed methods on large scales may be particularly desirable, due to the potential savings in time and resources.

Non-mammalian, e.g., microbial, cells can naturally produce, or can be engineered to produce, proteins that are expressed in either a soluble or a limited solubility form. Most often, engineered non-mammalian cells will deposit the recombinant proteins into large limited solubility aggregates called inclusion bodies. However, certain cell growth conditions (e.g., temperature or pH) can be modified to drive the recombinant proteins to be expressed as intracellular, soluble monomers. As an alternative to producing a protein of interest in cells in which the protein is expressed in the form of limited solubility inclusion bodies, cell growth conditions can be modified such that proteins are expressed in a non-native yet soluble form. The cells can then be lysed and the protein can be isolated by capturing it directly from cell lysate using ion exchange chromatography, affinity chromatography or mixed mode chromatography, as described herein. The method can be particularly useful for purifying proteins comprising an Fc region.

In one aspect, therefore, the present disclosure relates to a method of isolating a protein of interest comprising an Fc region that is expressed in a non-mammalian cell in a non-native, yet soluble form, from a pool of lysate generated from the cell in which the protein was expressed. The method employs a separation matrix, such as Protein A. One beneficial aspect of the disclosed method is that it eliminates the need for a refolding step before the protein is applied to the separation matrix. That is, non-mammalian cells expressing the protein of interest in a non-native soluble form can be lysed, the lysate applied directly to the separation matrix and the protein subsequently eluted from the separation matrix. This process allows the separation of proteins from cell cultures in highly concentrated pools that can be subsequently refolded at high concentrations and can be of benefit when producing large quantities of protein, particularly since the method is scalable from bench scale, which involves cultures on the order of several liters, up to production scale, which involves cultures of thousands of liters.

Following isolation by the separation matrix, the protein of interest can optionally be subsequently refolded using any technique known or suspected to work well for the protein of interest.

In another aspect, the present invention relates to a method of isolating a protein of interest comprising an Fc region that is expressed in a non-native limited solubility form, for example in inclusion bodies, that needs to be refolded and isolated from the refold mixture. Commonly, a refold solution contains a denaturant (e.g., urea or other chaotrope, organic solvent or strong detergent), an aggregation suppressor (e.g., a mild detergent, arginine or low concentrations of PEG), a protein stabilizer (e.g., glycerol, sucrose or other osmolyte, salts) and/or a redox component (e.g., cysteine, cystine, cystamine, cysteamine, glutathione). While often beneficial for refolding proteins, these components can inhibit purification (see, e.g., Wang et al., (1997) *Biochemical Journal* 325 (Part 3):707-710) and it is necessary to isolate or dilute the protein from these components for further processing, particularly before applying the protein to a separation matrix.

In one embodiment of the disclosed method, purification is achieved by directly applying a protein of interest, which is present in a refold mixture, to a separation matrix. In this approach, following a refold step the entire refold mixture, including the protein of interest, is applied directly to a separation matrix, such as a Protein A or G resin. The protein of interest associates with the matrix in the presence of the components of refold buffer, impurities are washed away and the protein is eluted. Since the method omits the need for removing any components of the refold mixture before the

refold mixture is applied to a separation matrix, the method can have the effect of saving steps, time and resources that are typically expended on removing the protein from refolding and dilution buffers in purification processes. In some cases, the method can also reduce or eliminate the need for subsequent purification steps.

The disclosed methods can also be employed to purify proteins expressed in a non-native soluble and non-native limited solubility forms in a non-mammalian expression system that have subsequently been derivatized. For example, following expression a protein comprising an Fc region can be associated with a small molecule, such as a toxin. Such conjugates can be purified using the methods described herein.

I. DEFINITIONS

As used herein, the terms "a" and "an" mean one or more unless specifically indicated otherwise.

As used herein, the term "non-mammalian expression system" means a system for expressing proteins in cells derived from an organism other than a mammal, including but not limited to, prokaryotes, including bacteria such as *E. coli*, and yeast. Often a non-mammalian expression system is employed to express a recombinant protein of interest, while in other instances a protein of interest is an endogenous protein that is expressed by a non-mammalian cell. For purposes of the present disclosure, regardless of whether a protein of interest is endogenous or recombinant, if the protein is expressed in a non-mammalian cell then that cell is a "non-mammalian expression system." Similarly, a "non-mammalian cell" is a cell derived from an organism other than a mammal, examples of which include bacteria or yeast.

As used herein, the term "denaturant" means any compound having the ability to remove some or all of a protein's secondary and tertiary structure when placed in contact with the protein. The term denaturant refers to particular chemical compounds that affect denaturation, as well as solutions comprising a particular compound that affect denaturation. Examples of denaturants that can be employed in the disclosed method include, but are not limited to urea, guanidinium salts, dimethyl urea, methylurea, ethylurea and combinations thereof.

As used herein, the term "aggregation suppressor" means any compound having the ability to disrupt and decrease or eliminate interactions between two or more proteins. Examples of aggregation suppressors can include, but are not limited to, amino acids such as arginine, proline, and glycine; polyols and sugars such as glycerol, sorbitol, sucrose, and trehalose; surfactants such as, polysorbate-20, CHAPS, Triton X-100, and dodecyl maltoside; and combinations thereof.

As used herein, the term "protein stabilizer" means any compound having the ability to change a protein's reaction equilibrium state, such that the native state of the protein is improved or favored. Examples of protein stabilizers can include, but are not limited to, sugars and polyhedric alcohols such as glycerol or sorbitol; polymers such as polyethylene glycol (PEG) and α-cyclodextrin; amino acids salts such as arginine, proline, and glycine; osmolytes and certain Hoffmeister salts such as Tris, sodium sulfate and potassium sulfate; and combinations thereof.

As used herein, the terms "Fc" and "Fc region" are used interchangeably and mean a fragment of an antibody that comprises human or non-human (e.g., murine) C_{H2} and C_{H3} immunoglobulin domains, or which comprises two contiguous regions which are at least 90% identical to human or non-human C_{H2} and C_{H3} immunoglobulin domains. An Fc can but need not have the ability to interact with an Fc receptor. See, e.g., Hasemann & Capra, "Immunoglobulins: Structure and Function," in William E. Paul, ed., *Fundamental Immunology*, Second Edition, 209, 210-218 (1989), which is incorporated by reference herein in its entirety.

As used herein, the terms "protein" and "polypeptide" are used interchangeably and mean any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds.

As used herein, the term "complex molecule" means any protein that is (a) larger than 20,000 MW, or comprises greater than 250 amino acid residues, and (b) comprises two or more disulfide bonds in its native form. A complex molecule can, but need not, form multimers. Examples of complex molecules include but are not limited to, antibodies, peptibodies and polypeptides comprising an Fc domain and other large proteins. Peptibodies are described in U.S. Pat. No. 6,660,843, U.S. Pat. No. 7,138,370 and U.S. Pat. No. 7,511,012.

As used herein, the term "peptibody" refers to a polypeptide comprising one or more bioactive peptides joined together, optionally via linkers, with an Fc domain. See U.S. Pat. No. 6,660,843, U.S. Pat. No. 7,138,370 and U.S. Pat. No. 7,511,012 for examples of peptibodies.

As used herein, the terms "Fc fusion" and "Fc fusion protein" are used interchangeably and refer to a peptide or polypeptide covalently attached to an Fc domain.

As used herein the term "Protein A" means any protein identical or substantially similar to Staphylococcal Protein A, including commercially available and/or recombinant forms of Protein A. For the purposes of this invention, Protein A specifically includes engineered Protein A derived media, such as Mab Select SuRe™ media (GE Healthcare), in which a single subunit (e.g., the B subunit) is replicated two or more times and joined in a contiguous sequence to form a recombinant Protein A molecule, and other non-naturally occurring Protein A molecules.

As used herein, the term "Protein G" means any protein identical or substantially similar to Streptococcal Protein G, including commercially available and/or recombinant forms of Protein G.

As used herein, the term "substantially similar," when used in the context of a protein, including Protein A, means proteins that are at least 80%, preferably at least 90% identical to each other in amino acid sequence and maintain or alter in a desirable manner the biological activity of the unaltered protein. Included in amino acids considered identical for the purpose of determining whether proteins are substantially similar are amino acids that are conservative substitutions, unlikely to affect biological activity, including the following: Ala for Ser, Val for Ile, Asp for Glu, Thr for Ser, Ala for Gly, Ala for Thr, Ser for Asn, Ala for Val, Ser for Gly, Tyr for Phe, Ala for Pro, Lys for Arg, Asp for Asn, Leu for Ile, Leu for Val, Ala for Glu, Asp for Gly, and these changes in the reverse. See, e.g., Neurath et al., *The Proteins*, Academic Press, New York (1979). The percent identity of two amino sequences can be determined by visual inspection and mathematical calculation, or more preferably, the comparison is done by comparing sequence information using a computer program such as the Genetics Computer Group (GCG; Madison, Wis.) Wisconsin package version 10.0 program, "GAP" (Devereux et al., 1984, *Nucl. Acids Res.* 12: 387) or other comparable computer programs. The preferred default parameters for the "GAP" program includes: (1) the weighted amino acid comparison matrix of

Gribskov and Burgess ((1986), *Nucl. Acids Res.* 14: 6745), as described by Schwartz and Dayhoff, eds., *Atlas of Polypeptide Sequence and Structure,* National Biomedical Research Foundation, pp. 353-358 (1979), or other comparable comparison matrices; (2) a penalty of 30 for each gap and an additional penalty of 1 for each symbol in each gap for amino acid sequences; (3) no penalty for end gaps; and (4) no maximum penalty for long gaps. Other programs used by those skilled in the art of sequence comparison can also be used.

As used herein, the terms "isolate" and "purify" are used interchangeably and mean to reduce by 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or more, the amount of heterogenous elements, for example biological macromolecules such as proteins or DNA, that may be present in a sample comprising a protein of interest. The presence of heterogenous proteins can be assayed by any appropriate method including High-performance Liquid Chromatography (HPLC), gel electrophoresis and staining and/or ELISA assay. The presence of DNA and other nucleic acids can be assayed by any appropriate method including gel electrophoresis and staining and/or assays employing polymerase chain reaction.

As used herein, the term "separation matrix" means any adsorbent material that utilizes specific, reversible interactions between synthetic and/or biomolecules, e.g., the property of Protein A to bind to an Fc region of an IgG antibody or other Fc-containing protein, in order to effect the separation of the protein from its environment. In other embodiments the specific, reversible interactions can be base on a property such as isoelectric point, hydrophobicity, or size. In one particular embodiment, a separation matrix comprises an adsorbent, such as Protein A, affixed to a solid support. See, e.g., Ostrove (1990) in "Guide to Protein Purification," *Methods in Enzymology* 182: 357-379, which is incorporated herein in its entirety.

As used herein, the terms "non-native" and "non-native form" are used interchangeably and when used in the context of a protein of interest, such as a protein comprising a Fc domain, mean that the protein lacks at least one formed structure attribute found in a form of the protein that is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity. Examples of structural features that can be lacking in a non-native form of a protein can include, but are not limited to, a disulfide bond, quaternary structure, disrupted secondary or tertiary structure or a state that makes the protein biologically inactive in an appropriate assay. A protein in a non-native form can but need not form aggregates.

As used herein, the term "non-native soluble form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, means that the protein lacks at least one formed structure attribute found in a form of the protein that is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity, but in which the protein is expressed in a form or state that is soluble intracellularly (for example in the cell's cytoplasm) or extracellularly (for example, in a lysate pool).

As used herein, the term "non-native limited solubility form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, means any form or state in which the protein lacks at least one formed structural feature found in a form of the protein that (a) is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity and/or (b) forms aggregates that require treatment, such as chemical treatment, to become soluble. The term specifically includes proteins existing in inclusion bodies, such as those sometimes found when a recombinant protein is expressed in a non-mammalian expression system.

As used herein, the term "soluble form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, broadly refers to a form or state in which the protein is expressed in a form that is soluble in a intracellularly (for example in the cell's cytoplasm) or extracellularly (for example, in a cell lysate pool).

II. DIRECT CAPTURE OF A PROTEIN EXPRESSED IN A NON-NATIVE SOLUBLE FORM IN A NON-MAMMALIAN EXPRESSION SYSTEM

One advantage of the disclosed method over typical purification methods is the elimination of the need for a refolding step before the soluble protein is applied to the separation matrix. That is, a protein solublized in cell lysate can be directly applied to the separation matrix. This is advantageous because the method does not require any initial purification efforts, although an initial filtration step may be desirable in some cases.

In the case of a protein comprising a Fc domain, the Fc region must have a certain level of structure to be bound by protein A, (Wang et al., (1997) *Biochem. J.* 325(Part 3):707-710). This fact has limited the application of separation matrices for purifying proteins that are expressed in a non-native soluble form, particularly proteins comprising an Fc region, because it is commonly believed that a soluble non-native Fc-containing protein would not have the requisite structural elements required to associate with a separation matrix. Furthermore, the Fc region of an antibody spontaneously forms a homodimer under non-reducing conditions and prior to the instant disclosure it was unexpected to observe that even in the reductive environment of the cell, the Fc-conjugated proteins and peptides not only form enough structure for protein to bind to the affinity resin, but that the individual peptide chains readily formed non-covalent dimers, even though the proteins had not yet been completely refolded to native form.

In view of prevailing beliefs, the success of the disclosed method was surprising and unanticipated because it was not expected that a non-mammalian, microbial cell fermentation could be induced to produce a protein that was soluble, yet still had enough structure to associate with the affinity separation matrix.

The disclosed method can be employed to purify a protein of interest that is expressed in a non-native soluble form in a non-mammalian cell expression system. The protein of interest can be produced by living host cells that either naturally produce the protein or that have been genetically engineered to produce the protein. Methods of genetically engineering cells to produce proteins are known in the art. See, e.g., Ausabel et al., eds. (1990), *Current Protocols in Molecular Biology* (Wiley, New York). Such methods include introducing nucleic acids that encode and allow expression of the protein into living host cells. In the context of the present disclosure, a host cell will be a non-mammalian cell, such as bacterial cells, fungal cells, yeast cells, and insect cells. Bacterial host cells include, but are not limited to, *Escherichia coli* cells. Examples of suitable *E. coli* strains include: HB101, DH5α, GM2929, JM109, KW251, NM538, NM539, and any *E. coli* strain that fails to cleave foreign DNA. Fungal host cells that can be used include, but are not limited to, *Saccharomyces cerevisiae, Pichia pasto-*

ris and *Aspergillus* cells. New cell lines can be established using methods known to those skilled in the art (e.g., by transformation, viral infection, and/or selection). It is noted that the method can be performed on proteins that are endogenously expressed by the non-mammalian cell as well.

During the production of a non-mammalian culture, growth conditions can be identified and employed so as to favor the production of a protein of interest in an intracellular soluble form. Such conditions can be identified by systematic empirical optimization of the culture condition parameters, such as temperature or pH. This optimization can be achieved using analysis of multifactorial matrices. For example, a matrix or series of multifactorial matrices can be evaluated to optimize temperature and pH conditions favor production of a desired species (i.e., a non-native soluble form). An optimization screen can be set up to systematically evaluate temperature and pH in a full or partial factorial matrix, with each component varied over a range of at least three temperature or pH levels with all other parameters kept constant. The protein can be expressed and the yield and quality of protein expressed in the desired form can be evaluated using standard multivariate statistical tools.

Initially, non-mammalian cells that express a particular protein of interest are grown to a desired target density under conditions designed to induce expression of the protein in a soluble form. In one embodiment, the cells express a wild type protein of interest. In another embodiment, the cells can be engineered using standard molecular biology techniques to recombinantly express a protein of interest, and induced to produce the protein of interest. The protein of interest can be any protein, for example a protein that comprises an Fc moiety. Such a protein can be, for example, an antibody, a peptibody or an Fc fusion protein, any of which can be joined to an Fc moiety via a linker.

Once the desired target density is reached, the non-mammalian cells are separated from the growth media. One convenient way of achieving separation is by centrifugation, however filtration and other clarification methods can also be used.

The cells are then collected and are resuspended to an appropriate volume in a resuspension solution. Examples of resuspension solutions that can be used in the disclosed methods include phosphate buffered saline, Tris buffered saline, or water. The selection of an appropriate buffer will be determined, in part, by the properties of the molecule of interest as well as any volume or concentration constraints.

Following resuspension, the non-mammalian cells are lysed to release the protein, which will be present in the cell lysate in a non-native soluble form to generate a cell lysate. The lysis can be performed using any convenient means, such as feeding the cell suspension through a high pressure homogenizer or by employing a chemical lysis process. Whichever lytic process is selected, the function of the lysis step is to break open the cells and to break down DNA. The lysis can be performed in multiple cycles to achieve a more complete lysis or to accommodate large volumes of cell suspension. For example, the cell suspension can be fed through a mechanical homogenizer several times. This process releases the intracellular contents, including the protein of interest, and forms a pool of cell lysate.

Following the lysis procedure, the cell lysate can optionally be filtered. Filtration can remove particulate matter and/or impurities, such as nucleic acids and lipids, and may be desirable in some cases, such as when one suspects that direct application of the cell lysate to the chromatography equipment or media may lead to fouling or clogging, or when the separation matrix is sensitive to fouling or difficult to clean in-place. The benefit of filtering the cell lysate prior to contacting it with the separation matrix can be determined on a case-by-case basis.

After the lysis procedure, the cell lysate can optionally be incubated for an appropriate amount of time in the presence of air or oxygen, or exposed to a redox component or redox thiol-pair. The incubation can facilitate and/or ensure the formation of the minimal secondary structure required to facilitate an association with a separation matrix. The particular length of the incubation can vary with the protein but is typically less than 72 hours (e.g., 0, 0.5, 1, 2, 3, 5, 7, 10, 12, 18, 24, 36, 48 or 72 hours). When an incubation is performed, the length of incubation time can be determined by empirical analysis for each protein, which in some cases will be shorter (or omitted) and other cases longer.

Following the incubation period the cell lysate, which comprises the released protein of interest, is contacted with a separation matrix under conditions suitable for the protein to associate with a binding element of the separation matrix. Representative conditions conducive to the association of a protein with an affinity matrix are provided in the Examples. The separation matrix can be any media by which the protein of interest can be separated from the components of the resuspension and/or lysis buffer, including impurities such as host cell proteins, DNA, lipids and chemical impurities introduced by the components of the resuspension and/or lysis buffer.

Proteins A and G are often employed to purify antibodies, peptibodies and other fusion proteins comprising a Fc region by affinity chromatography. See, e.g., Vola et al. (1994), *Cell Biophys.* 24-25: 27-36; Aybay and Imir (2000), *J. Immunol. Methods* 233(1-2): 77-81; Ford et al. (2001), *J. Chromatogr. B* 754: 427-435. Proteins A and G are useful in this regard because they bind to the Fc region of these types of proteins. Recombinant fusion proteins comprising an Fc region of an IgG antibody can be purified using similar methods. Proteins A and G can be employed in the disclosed methods as an adsorbent component of a separation matrix.

Thus, examples of separation matrices that can be employed in the present invention include Protein A resin, which is known to be, and is commonly employed as, an effective agent for purifying molecules comprising an Fc moiety, as well as Protein G and synthetic mimetic affinity resins, such as MEP HyperCel® chromatography resin.

After the protein of interest has been associated with the separation matrix by contacting the cell lysate containing the protein with the separation matrix, thereby allowing the protein to associate with the adsorbent component of the separation matrix, the separation matrix is washed to remove unbound lysate and impurities.

The wash buffer can be of any composition, as long as the composition and pH of the wash buffer is compatible with both the protein and the matrix, and maintains the interaction between the protein and the matrix. Examples of suitable wash buffers that can be employed include solutions containing glycine, Tris, citrate, or phosphate; typically at levels of 5-100 mM (e.g., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75 or 100 mM). These solutions can also contain an appropriate salt ion, such as chloride, sulfate or acetate at levels of 5-500 mM (e.g., 5, 10, 12, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450 or 500 mM). The resin can be washed once or any number of times. The exact composition of a wash buffer will vary with the protein being purified.

After the separation matrix with which the protein has associated has been washed, the protein of interest is eluted from the matrix using an appropriate solution. The protein of interest can be eluted using a solution that interferes with the

binding of the adsorbent component of the separation matrix to the protein, for example by disrupting the interactions between the separation matrix and the protein of interest. This solution can include an agent that can either increase or decrease pH, and/or a salt. For example, the pH can be lowered to about 4.5 or less, for example to between about 3.3 and about 4.0, e.g., 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4 or 4.5. A solution comprising citrate or acetate, for example, can be employed to lower the pH. Other methods of elution are also known, such as via the use of chaotropes (see, e.g., Ejima et al. (2005) *Analytical Biochemistry* 345(2):250-257) or amino acid salts (see, e.g., Arakawa et al. (2004) *Protein Expression & Purification* 36(2):244-248). Protocols for such affinity chromatography are well known in the art. See, e.g., Miller and Stone (1978), *J. Immunol. Methods* 24(1-2): 111-125. Conditions for binding and eluting can be readily optimized by those skilled in the art. The exact composition of an elution buffer will vary with the protein being purified. The protein can then optionally be further purified from the elution pool and refolded as necessary. In other situations the protein need not be further purified and instead can be refolded directly from the elution pool. Refolding directly from the elution pool may or may not require denaturation or reduction of the protein prior to incubation in a refolding solution and will depend in part on the properties of the protein.

In some cases it will be desirable to provide the separation matrix in a column format. In such cases a chromatography column can be prepared and then equilibrated before the cell suspension is loaded. Techniques for generating a chromatography column are well known and can be employed. An optional preparation and equilibration step can comprise washing the column with a buffer having an appropriate pH and salt condition that is conducive to protein-matrix interactions. This step can provide the benefit of removing impurities present in the separation matrix and can enhance the binding of the protein to be isolated to the adsorbent component of a separation matrix.

As noted, the separation matrix can be disposed in a column. The column can be run with or without pressure and from top to bottom or bottom to top. The direction of the flow of fluid in the column can be reversed during the purification process. Purifications can also be carried out using a batch process in which the solid support is separated from the liquid used to load, wash, and elute the sample by any suitable means, including gravity, centrifugation, or filtration. Moreover, purifications can also be carried out by contacting the sample with a filter that adsorbs or retains some molecules in the sample more strongly than others, such as anion exchange membrane chromatography.

If desired, the protein concentration of a sample at any given step of the disclosed method can be determined, and any suitable method can be employed. Such methods are well known in the art and include: 1) colorimetric methods such as the Lowry assay, the Bradford assay, the Smith assay, and the colloidal gold assay; 2) methods utilizing the UV absorption properties of proteins; and 3) visual estimation based on stained protein bands on gels relying on comparison with protein standards of known quantity on the same gel. See, e.g., Stoschek (1990), "Quantitation of Protein," in "Guide to Protein Purification," *Methods in Enzymology* 182: 50-68. Periodic determinations of protein concentration can be useful for monitoring the progress of the method as it is performed.

It is noted that any or all steps of the disclosed methods can be carried out manually or by any convenient automated means, such as by employing automated or computer-controlled systems.

III. DIRECT CAPTURE OF NON-NATIVE LIMITED SOLUBILITY PROTEIN FORMS FROM A REFOLD SOLUTION FOLLOWING EXPRESSION IN NON-MAMMALIAN CELLS

In another aspect of the present disclosure, a method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system is disclosed. An advantage of the disclosed method is that the method eliminates the need for removing or diluting the refold solution before applying the protein to a separation matrix, thereby saving the time and resources associated with what is a typical step in a purification process for isolating proteins expressed in a non-native limited solubility form.

Non-mammalian cells, e.g., microbial cells, can produce recombinant proteins that are expressed intracellularly in either a soluble or a limited solubility form. When the growth conditions are not directed to force expression of the protein in a soluble form, the cells may deposit the recombinant proteins into large relatively insoluble aggregates, such as inclusion bodies. These aggregates comprise protein that is typically not biologically active or less active than the completely folded native form of the protein. In order to produce a functional protein, these inclusion bodies often need to be carefully denatured so that the protein of interest can be extracted and refolded into a biologically active form.

In typical approaches, the inclusion bodies need to be captured, washed, exposed to a denaturing and/or reducing solubilization solution and the denaturing solution is then diluted with a solution to generate a condition that allows the protein to refold into an active form and form a structure that is found in the native protein. Subsequently, it is necessary to remove the components of the diluted denaturing solution from the immediate location of the protein. In order to do this, the refold solution comprising the solubilization solution and the refolded protein is typically diluted with a buffered solution before it is applied to a separation matrix, such as a Protein A ion exchange or other mixed-mode adsorbents. This process can be time-consuming and resource-intensive. It also significantly increases the volumes that need to be handled, as well as the associated tankage requirements, which can become limiting when working on large scales. The disclosed method eliminates the need for such a dilution step

The disclosed method is particularly useful for purifying a protein of interest that is expressed in a non-native limited solubility form in a non-mammalian cell expression system. The protein of interest can be produced by living host cells that either naturally produce the protein or that have been genetically engineered to produce the protein. Methods of genetically engineering cells to produce proteins are well known in the art. See, e.g., Ausabel et al., eds. (1990), *Current Protocols in Molecular Biology* (Wiley, New York). Such methods include introducing nucleic acids that encode and allow expression of the protein into living host cells. In the context of the present disclosure, these host cells will be non-mammalian cells, such as bacterial cells, fungal cells. Bacterial host cells include, but are not limited to *Escherichia coli* cells. Examples of suitable *E. coli* strains include: HB101, DH5α, GM2929, JM109, KW251, NM538, NM539, and any *E. coli* strain that fails to cleave foreign

DNA. Fungal host cells that can be used include, but are not limited to, *Saccharomyces cerevisiae, Pichia pastoris* and *Aspergillus* cells. New cell lines can be established using methods well know by those skilled in the art (e.g., by transformation, viral infection, and/or selection). It is noted that the method can be performed on endogenous proteins that are naturally expressed by the non-mammalian cell as well.

Initially, non-mammalian cells that express a particular protein of interest are grown to a desired target density. In one embodiment, the cells can be expressing a particular wild type microbial protein of interest. In another embodiment, the cells can be engineered using standard molecular biology techniques to recombinantly express a protein of interest, and in this context they can be induced to overproduce the protein of interest. The protein of interest can be any protein, for example a protein that comprises an Fc moiety. Such a protein can be, for example, an antibody, a peptibody or an Fc fusion protein, any of which can be joined to an Fc moiety via a linker.

Once the desired target density is reached, the non-mammalian cells can be separated from the growth media. One convenient way of achieving separation is by centrifugation, however filtration and other clarification methods can also be used.

The cells are then collected and are resuspended to an appropriate volume in a resuspension solution. Examples of resuspension solutions that can be used in the present invention include phosphate-buffered saline, Tris-buffered saline, or water. The selection of an appropriate buffer will be determined, in part, by the properties of the molecule of interest as well as any volume or concentration constraints.

In order to release the limited solubility non-native protein from the cells, the non-mammalian cells are lysed to form a cell lysate comprising the released the limited solubility non-native protein. The lysis can be performed in any convenient way, such as feeding the cell suspension through a high pressure homogenizer or by employing a chemical lysis process. Whichever lysis process is selected, the function of the lysis step is to break open the cells and to break down DNA. The lysis can be performed in multiple cycles to achieve a more complete lysis or to accommodate large volumes of cell suspension. For example, the cell suspension can be fed through a mechanical homogenizer several times. This process releases the intracellular contents, including the naturally-occurring or recombinant protein of interest, and forms a pool of cell lysate.

Next, the limited solubility non-native protein is separated from the rest of the lysis pool. This can be done, for example, by centrifugation. Representative conditions for a centrifuge-mediated separation or washing typically include removal of excess water from the cell lysate, resuspension of the resulting slurry in a resuspension solution. This washing process may be performed once or multiple times. Examples of typical centrifuge types include, but are not limited to, disk-stack, continuous discharge, and tube bowl. Examples of resuspension solutions that can be used in the present invention include phosphate-buffered saline, Tris-buffered saline, or water and can include other agents, such as ETDA or other salts. The selection of an appropriate buffer will be determined, in part, by the properties of the molecule of interest as well as any volume or concentration constraints. The exact composition of an resuspension buffer will vary with the protein being purified.

The expressed protein is then solubilized in a solubilization solution comprising one or more of (i) a denaturant, (ii) a reductant and (iii) a surfactant. The denaturant can be included as a means of unfolding the limited solubility protein, thereby removing any existing structure, exposing buried residues and making the protein more soluble.

Any denaturant can be employed in the solubilization solution. Examples of some common denaturants that can be employed in the refold buffer include urea, guanidinium, dimethyl urea, methylurea, or ethylurea. The specific concentration of the denaturant can be determined by routine optimization.

The reductant can be included as a means to reduce exposed residues that have a propensity to form covalent intra or intermolecular-protein bonds and minimize non-specific bond formation. Examples of suitable reductants include, but are not limited to, cysteine, DTT, beta-mercaptoethanol and glutathione. The specific concentration of the reductant can be determined by routine optimization.

A surfactant can be included as a means of unfolding the limited solubility non-native protein, thereby exposing buried residues and making the protein more soluble. Examples of suitable surfactants include, but are not limited to, sarcosyl and sodium dodecylsulfate. The specific concentration of the surfactant can be determined by routine optimization.

Although the composition of a solubilization solution will vary with the protein being purified, in one particular embodiment the solubilization solution comprises 4-6 M guanidine, 50 mM DTT.

Continuing, a refold solution comprising the solubilization solution (which comprises the protein), and a refold buffer is formed. The refold buffer comprises one or more of (i) a denaturant; (ii) an aggregation suppressor; (iii) a protein stabilizer; and (iv) a redox component. The denaturant can be included as a means of modifying the thermodynamics of the solution, thereby shifting the equilibrium towards an optimal balance of native form. The aggregation suppressor can be included as a means of preventing non-specific association of one protein with another, or with one region of a protein with another region of the same protein. The protein stabilizer can be included as a means of promoting stable native protein structure and may also suppress aggregation.

In various embodiments, the denaturant in the refold buffer can be selected from the group consisting of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea.

In various embodiments, the protein stabilizer in the refold buffer can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

In various embodiments, the aggregation suppressor can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

In various embodiments, the thiol-pairs can comprise at least one component selected from the group consisting of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

The specific concentrations of the components of a refold buffer can be determined by routine optimization. For example, a matrix or series of multifactorial matrices can be evaluated to optimize the refolding buffer for conditions that optimize yield and distributions of desired species. An optimization screen can be set up to systematically evaluate denaturant, aggregation suppressor, protein stabilizer and redox component concentrations and proportions in a full or partial factorial matrix, with each component varied over a

range of concentrations with all other parameters kept constant. The completed reactions can be evaluated by RP-HPLC and SE-HPLC analysis for yield and product quality using standard multivariate statistical tools.

The function of the buffer component of the refold solution is to maintain the pH of the refold solution and can comprise any buffer that buffers in the appropriate pH range. Examples of the buffering component of a refold buffer that can be employed in the method include, but are not limited to, phosphate buffers, citrate buffers, tris buffer, glycine buffer, CHAPS, CHES, and arginine-based buffers, typically at levels of 5-100 mM (e.g., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 80, 85, 90, 95 or 100, mM).

Although the composition of an refold buffer will vary with the protein being purified, in one embodiment a refold buffer comprises arginine, urea, glycerol, cysteine and cystamine.

The refold solution can then be incubated for a desired period of time. The incubation period can be of any length but is typically between 0 and 72 hours (e.g., 0, 0.5, 1, 2, 3, 5, 7, 10, 12, 18, 24, 36, 48 or 72 hours).

After an appropriate incubation time, the refold solution is then applied to a separation matrix under conditions suitable for the protein to associate with the matrix. The separation matrix can be any media by which the protein of interest can be separated from the components of the resuspension and/or lysis buffer, including impurities such as host cell proteins, DNA and chemical impurities introduced by the components of the solubilization and/or lysis buffer.

Proteins A and G are often employed to purify antibodies, peptibodies and other fusion proteins comprising a Fc region by affinity chromatography. See, e.g., Vola et al. (1994), *Cell Biophys*. 24-25: 27-36; Aybay and Imir (2000), *J. Immunol. Methods* 233(1-2): 77-81; Ford et al. (2001), *J. Chromatogr. B* 754: 427-435. Proteins A and G are useful in this regard because they bind to the Fc region of these types of proteins. Recombinant fusion proteins comprising an Fc region of an IgG antibody can be purified using similar methods. Proteins A and G can be employed in the disclosed methods as an adsorbent component of a separation matrix.

Thus, examples of affinity separation matrices that can be employed in the present invention include Protein A resin, which is know to be, and is commonly employed as, an effective agent for purifying molecules comprising an Fc moiety, as well as Protein G and synthetic mimetic affinity resins. Other materials that can be employed include HIC and ion exchange resins (see Example 4), depending on the properties of the protein to be purified.

It is noted that when performing the method, the refold solution comprising the refolded protein of interest is applied directly to the separation matrix, without the need for diluting or removing the components of the solution required for refolding the protein. This is an advantage of the disclosed method. Initially, it was expected that the highly ionic and/or chaotropic compounds and various other components of the refold solution would inhibit the association of the protein with the separation matrix. However, in contrast to reports in the literature (e.g., Wang et al. (1997) *Biochemical Journal*. 325(Part 3):707-710), it was surprising to observe that the protein was in fact able to associate with the separation matrix in the presence of the components of the refold solution. The unexpected finding that the protein could associate with the separation matrix in the presence of the components of the refold solution facilitates the elimination of a dilution step or buffer exchange operation, providing a savings of time and resources.

After the protein of interest has associated with the separation matrix the separation matrix is washed to remove unbound protein, lysate, impurities and unwanted components of the refold solution.

The wash buffer can be of any composition, as long as the composition and pH of the wash buffer is compatible with both the protein and the matrix. Examples of suitable wash buffers that can include, but are limited to, solutions containing glycine, tris, citrate, or phosphate. These solutions may also contain an appropriate salt. Suitable salts include, but are not limited to, sodium, potassium, ammonium, magnesium, calcium, chloride, fluoride, acetate, phosphate, and/or citrate. The pH range is chosen to optimize the chromatography conditions, preserve protein binding, and to retain the desired characteristics of the protein of interest. The resin can be washed once or any number of times. The exact composition of a wash buffer will vary with the protein being purified.

After the separation matrix with which the protein has associated has been washed, the protein of interest is eluted using an appropriate solution (e.g., a low pH buffered solution or a salt solution) to form an elution pool comprising the protein of interest.

The protein of interest can be eluted using a solution that interferes with the binding of the adsorbent component of the separation matrix to the protein, for example by disrupting the interactions between Protein A and the Fc region of a protein of interest. This solution may include an agent that can either increase or decrease pH, and/or a salt. In various embodiments, the elution solution can comprise acetic acid, glycine, or citric acid. Elution can be achieved by lowering the pH. For example, the pH can be lowered to about 4.5 or less, for example to between about 3.3 to about 4.2 (e.g., 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1 or 4.2, using a solution comprising citrate or acetate, among other possibilities.

In some situations, the protein can then be further purified from the elution pool and can be further refolded, if necessary. In other situations the protein need not be further purified and instead can be further refolded directly in the elution pool, if necessary.

Protocols for such affinity chromatography are known in the art. See, e.g., Miller and Stone (1978), *J. Immunol. Methods* 24(1-2): 111-125. In the cases that utilize ion exchange, mixed-mode, or hydrophobic interaction chromatography, the concentration of salt can be increased or decreased to disrupt ionic interaction between bound protein and a separation matrix. Solutions appropriate to effect such elutions can include, but are not limited to, sodium, potassium, ammonium, magnesium, calcium, chloride, fluoride, acetate, phosphate, and/or citrate. Other methods of elution are also known. Conditions for binding and eluting can be readily optimized by those skilled in the art.

The exact composition of an elution buffer will vary with the protein being purified and the separation matrix being employed.

In some cases it will be desirable to situate the separation matrix in a column format. In such cases a column can be prepared and then equilibrated before the cell suspension is loaded. Techniques for generating a chromatography column are well known and can be employed. The optional preparation and equilibration step can comprise washing the column with a buffer having an appropriate pH and composition that will prepare the media to bind a protein of interest. This step has the benefit of removing impurities present in the separation matrix and can enhance the binding of the protein to be isolated to the adsorbent component of a separation matrix.

It is noted that any or all steps of the invention can be carried out by any mechanical means. As noted, the separation matrix can be disposed in a column. The column can be run with or without pressure and from top to bottom or bottom to top. The direction of the flow of fluid in the column can be reversed during the purification process. Purifications can also be carried out using a batch process in which the solid support is separated from the liquid used to load, wash, and elute the sample by any suitable means, including gravity, centrifugation, or filtration. Moreover, purifications can also be carried out by contacting the sample with a filter that adsorbs or retains some molecules in the sample more strongly than others.

If desired, the protein concentration of a sample at any given step of the disclosed method can be determined by any suitable method. Such methods are well known in the art and include: 1) colorimetric methods such as the Lowry assay, the Bradford assay, the Smith assay, and the colloidal gold assay; 2) methods utilizing the UV absorption properties of proteins; and 3) visual estimation based on stained protein bands on gels relying on comparison with protein standards of known quantity on the same gel. See, e.g., Stoschek (1990), "Quantitation of Protein," in "Guide to Protein Purification," *Methods in Enzymology* 182: 50-68. Periodic determinations of protein concentration can be useful for monitoring the progress of the method as it is performed.

It is noted that any or all steps of the disclosed methods can be carried out manually or by any convenient automated means, such as by employing automated or computer-controlled systems.

IV. COLUMN CLEANING

In another aspect the present disclosure relates to the observation that in many cases the separation matrix employed in the methods provided herein can be cleaned after multiple separations and reused. This unexpected property of the method provides a significant cost and resource savings, particularly on the manufacturing scale, since the separation matrix need not be discarded after a separation is complete.

Common wisdom in the industry suggests that after a separation matrix, such as Protein A, is repeatedly exposed to highly heterogenous feedstocks comprising high lipid and host protein content it becomes irreversibly contaminated and unusable when treated with the mild regeneration solutions commonly utilized for protein-based affinity resins. The disclosed methods, however, avoid this situation and extend the usable lifetime of a separation matrix. In the context of a large scale manufacturing process this can translate into a measurable savings of time and money. Moreover, the cleaning step can be performed, as disclosed in the Examples, in-place and with no need to extract the separation matrix from a column or other matrix retaining device for cleaning, thus saving time and resources.

In one embodiment of a cleaning operation of a separation matrix, following a separation employing the disclosed method the separation matrix is washed with a regeneration reagent, such as sodium hydroxide, or an acidic reagent, such as phosphoric acid.

In one particular embodiment of a cleaning operation, Protein A is the separation matrix and a column containing Protein A resin is washed with 5 column volumes of 150 mM phosphoric acid and held for >15 minutes over the column. Following the wash with the acid, the column can be flushed with water, regenerated with 5 column volumes of 50 mM Tris, 10 mM citrate, 6M urea, 50 mM DTT; pH 7.4, subsequently washed with water, and then flushed with 3 column volumes of 150 mM phosphoric acid. This cleaning protocol has been utilized to achieve over 200 cycles of protein A resin. FIG. **3** highlights the results achievable using the disclosed cleaning methods.

EXAMPLES

The following examples demonstrate embodiments and aspects of the present invention and are not intended to be limiting.

Example 1

Direct Capture of Proteins Expressed in a Soluble Form Using Protein A Affinity Chromatography

The following experiment demonstrates that a protein comprising a plurality of polypeptides joined to an Fc moiety can be separated from an *E. coli* cell lysate slurry using a Protein A affinity media.

A protein comprising a plurality of polypeptides joined to an Fc moiety was expressed in an *E. coli* fermentation induced at 30° C. and driven to express soluble-form protein product. The fermentation broth was centrifuged, the liquid fraction removed, and the cell paste was collected. The cells were resuspended in a 10 mM potassium phosphate, 5 mM EDTA; pH 6.8 buffer solution, to approximately 100% of the original volume. The cells were then lysed by means of three passes through a high pressure homogenizer. After the cells were lysed, the cell lysate was filtered through a 0.1 µm filter to reduce particulate levels. The material was then stored in a closed bottle for ~24 hours at approximately 5° C.

In a separate operation, a packed column comprising GE Healthcare Mab Select™ Protein A affinity resin was prepared and equilibrated with 5 column volumes (CV) of 10 mM Tris; pH 8.0.

An aliquot of a protein comprising an Fc moiety was sampled directly from a lysate. The protein mixture was loaded to approximately 0.02 millimoles total protein/L resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 10 mM Tris; pH 8.0, for 5 CV at up to 220 cm/hr. The protein of interest was recovered from the resin by elution with 50 mM sodium acetate, pH 3.1 at up to 220 cm/hr. The elution pool yielded greater than 90% recovery of the soluble material in the initial cell broth. The collected protein in the elution pool was stored at 2-8° C. until the next purification step was carried out.

Following the separation, the resin media was cleaned in-place by flowing 5 CV of 6 M Guanidine, pH 8.0 at 220 cm/hr.

The results of this separation demonstrated that a soluble protein expressed in a non-mammalian system can be captured and purified, with high yield, directly from cell lysate broth without having to refold the protein prior to application to a separation matrix.

Example 2

Capture of a Fc-Containing Protein Expressed in a Limited Solubility Form from a Refold Mixture Using Protein A Affinity Chromatography

The following experiments demonstrate that an Fc-containing protein can be separated from a refold mixture comprising glycerol, guanidine, urea, and arginine using Protein A affinity media.

In one experiment, a recombinant protein comprising a biologically active peptide linked to the C-terminus of the Fc moiety of an IgG1 molecule via a linker and having a molecular weight of about 57 kDa and comprising 8 disulfide bonds, in a non-mammalian expression system, namely *E. coli*, harvested, refolded under appropriate conditions, and captured using Protein A affinity media.

The growth media in which the cells were growing was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water to approximately 60% of the original volume. The cells were lysed by means of three passes through a high pressure homogenizer.

After the cells were lysed, the lysate was centrifuged in a disc-stack centrifuge to collect the protein in the solid fraction, which was expressed in a limited solubility non-native form, namely as inclusion bodies.

The protein slurry was washed multiple times by resuspending the slurry in water to between 50 and 80% of the original fermentation broth volume, mixing, and centrifugation to collect the protein in the solid fraction.

The concentrated protein was then combined in a solubilization solution containing the protein, guanidine, urea, and DTT.

After incubation for one hour, the protein solution was diluted in to a refold buffer containing appropriate levels of arginine, urea, glycerol, cysteine, and cystamine.

In a separate operation, a packed column comprising ProSep VA Ultra™ Protein A affinity resin with dimensions of 1.1 cm internal diameter and ~25 cm height, was prepared and equilibrated with 5 column volumes (CV) of 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution.

An aliquot of a protein comprising an Fc moiety from the refold solution was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was loaded to approximately 0.35 millimoles total protein/L resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution, for 4.5 CV at up to 400 cm/hr. The Fc-containing protein was recovered from the resin by elution with 100 mM sodium acetate, pH 3.7 at up to 300 cm/hr. The average level of purity achieved is shown in FIG. **3**.

Following the separation, the resin media was cleaned in-place by flowing 5 CV of 150 mM phosphoric acid. The column was regenerated with 5CV of 50 mM Tris, 10 mM citrate, 6M urea and 50 mM DTT; pH 7.4, washed with water, and then flushed with 3CV of 150 mM phosphoric acid.

The results of this separation demonstrate that an insoluble protein expressed in a non-mammalian system can be purified directly from a refold buffer without having to dilute the refold buffer prior to application to a separation matrix for more than 150 cycles, as indicated by the table presented in FIG. **3**.

In another separation, the Protein A column was cycled with the above procedure 8-10 times and then the final cycle was run as follows: The media was equilibrated with 5 column volumes (CV) of 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution. An aliquot of protein sampled directly from a refold buffer was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was then loaded on the column to 0.35 millimoles total protein/L resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution, for 4.5 CV at up to 400 cm/hr. The protein of interest was recovered from the resin by eluting with 100 mM sodium acetate, pH 3.7 at up to 300 cm/hr. The resin media was cleaned in-place by flowing 5 CV of 150 mM phosphoric acid over it. Finally, the column was flushed with water, regenerated with 5CV of 50 mM Tris, 10 mM citrate, 6M urea, and 50 mM DTT; pH 7.4, washed with water, and then flushed with 3CV of 150 mM phosphoric acid. Subsequent analysis of the resin showed no protein carry-over between cycles, demonstrating the ability to reuse the resin after both cleaning methods.

Example 3

Separation of an Fc-Containing Protein from a Refold Mixture Using Cation Exchange Chromatography

The following experiments demonstrate that an Fc-containing protein can be separated from a refold mixture comprising glycerol, guanidine, urea, and arginine using cation exchange media.

In one experiment, a recombinant protein comprising a biologically active peptide linked to the C-terminus of the Fc moiety of an IgG1 molecule via a linker and having a molecular weight of about 57 kDa and comprising 8 disulfide bonds, was expressed in a non-mammalian expression system, namely *E. coli*, harvested, refolded under appropriate conditions, and captured using cation exchange media.

The growth media in which the cells were growing was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water. The cells were lysed by means of multiple passes through a high pressure homogenizer. After the cells were lysed, the lysate was centrifuged to collect the protein, which was expressed in a limited solubility non-native form, namely as inclusion bodies. The protein slurry was washed multiple times by resuspending the slurry in water, mixing, and centrifugation to collect the protein. The concentrated protein was then transferred to a solubilization buffer containing guanidine and DTT. After incubation for one hour, the protein solution was diluted in to a refold buffer containing appropriate levels of arginine, urea, glycerol, cysteine, and cystamine.

In a separate operation, a packed column comprising EMD Fractogel $S0_3^-$ cation exchange resin with dimensions of 1.1 cm internal diameter and 20 cm height, was prepared and equilibrated with 5 column volumes of 30 mM MES; pH 4.5 buffered solution.

An aliquot of a protein comprising an Fc moiety was sampled directly from a refold solution, was diluted 3-fold with water, titrated with 50% hydrochloric acid to ~pH 4.5 and was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was loaded to approximately 0.96 millimoles total protein/L resin at 60 cm/hr.

After loading, the column was washed with 30 mM MES; pH 4.5, for 3 CV at 60 cm/hr, then washed with an additional 3 CV of 30 mM MES; pH 6.0. The protein of interest was recovered from the resin by gradient elution over 25 CV between 30 mM MES; pH 6.0 and 30 mM MES, 500 mM

NaCl; pH 6.0 at 60 cm/hr. The collected protein in the elution pool was stored at 2-8° C. until the next purification step was carried out.

Purity levels achieved, as determined by SEC and RP-HPLC are shown in FIG. **5**.

Following the separation, the resin media was cleaned in-place by flowing 3 CV of 1 M sodium hydroxide, at 120 cm/hr and held for 60 minutes prior an additional 3CV wash with 1 m sodium hydroxide.

The results of this separation demonstrate that an insoluble protein expressed in a non-mammalian system can be captured and purified from a refold buffer with a variety of separation matrices, including an ion-exchange separation matrix.

Example 4

Re-Usability of Protein A Affinity Resin Used to Isolate a Fc-Containing Protein Directly from a Refold Buffer by Affinity Chromatography

In another aspect of the method, a range of column cleaning methods can be employed in conjunction with the methods described herein, allowing the chromatography resins to be reused to an extent that make the method economically feasible. As described in Examples 2 and 3 for the case of Protein A affinity resins, cleaning protocols have been developed and demonstrated to remove product and non-product contaminants from the resin to allow reuse. The cleaning agents include caustic (e.g. sodium or potassium hydroxide), detergents (e.g. SDS or Triton X-100), denaturants (e.g. urea or guanidine-derivatives), and reductants (e.g. DTT, or thioglycolates). These agents can be used in combination or alone.

In order to demonstrate the reusability of column resins following application of the direct capture methods described, an aliquot of pH adjusted and filtered Fc-containing protein was loaded on new, unused resin and resin that had been previously cycled 94 times to evaluate the cleaning of the Protein A resin and the effect on purification binding and separation of an Fc-containing protein with regard to resin history.

The media was equilibrated with 5 column volumes (CV) of 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution. An aliquot of protein sampled directly from a refold buffer was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was then loaded on the column to approximately 0.35 millimoles total protein/mL resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution, for 4.5 CV at up to 400 cm/hr. The protein of interest was recovered from the resin by eluting with 100 mM sodium acetate, pH 3.7 at up to 300 cm/hr. Each column was regenerated using 5CV phosphoric acid and 5 CV of an acidic buffered solution containing 50 mM Tris, 10 mM citrate, 6M urea, and 50 mM DTT; pH 7.4.

This procedure was repeated for greater than 100 cycles. Selected samples from this reuse study were submitted for SEC-HPLC analysis. The goal was to track the % MP purity, % HMW and % dimer species from the pools as well as to understand the change of purity level from the load. No major differences were observed between the used columns and new columns.

This Example demonstrates that not only can a complex protein be captured from a complex chemical solution, but that the resin can be cycled repeatedly and cleaned and reused reproducibly over a number of industrially-relevant cycles.

What is claimed is:

1. A method of purifying a protein expressed in a non-native soluble form in a non-mammalian expression system comprising:
- (a) lysing a non-mammalian cell in which the protein is expressed in a nonnative soluble form to generate a cell lysate;
- (b) contacting the cell lysate with a separation matrix under conditions suitable for the protein to associate with the separation matrix;
- (c) washing the separation matrix; and
- (d) eluting the protein from the separation matrix.

2. The method of claim **1**, wherein the protein is a complex protein.

3. The method of claim **2**, wherein the complex protein is selected from the group consisting of a multimeric protein, an antibody and an Fc fusion protein.

4. The method of claim **1**, wherein the non-mammalian expression system comprises bacteria or yeast cells.

5. The method of claim **1**, wherein the separation matrix is an affinity resin.

6. The method of claim **1**, wherein the separation matrix is a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

7. The method of claim **1**, wherein the cell lysate is filtered before it is contacted with the separation matrix.

8. The method of claim **1**, further comprising refolding the protein to its native form after it is eluted.

9. A method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system comprising:
- (a) solubilizing the expressed protein in a solubilization solution comprising one or more of the following:
  - (i) a denaturant;
  - (ii) a reductant; and
  - (iii) a surfactant;
- (b) forming a refold solution comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of the following:
  - (i) a denaturant;
  - (ii) an aggregation suppressor;
  - (iii) a protein stabilizer; and
  - (iv) a redox component;
- (c) applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix;
- (d) washing the separation matrix; and
- (e) eluting the protein from the separation matrix.

10. The method of claim **9**, wherein the non-native limited solubility form is a component of an inclusion body.

11. The method of claim **9**, wherein the protein is a complex protein.

12. The method of claim **10**, wherein the complex protein is selected from the group consisting of a multimeric protein, an antibody, a peptibody, and an Fc fusion protein.

13. The method of any one of claims **9**-**12**, wherein the non-mammalian expression system comprises bacteria or yeast cells.

14. The method of any one of claims **9**-**12**, wherein the denaturant of the solubilization solution or the refold buffer

comprises one or more of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea.

15. The method of claim **9**, wherein the reductant comprises one or more of cysteine, dithiothreitol (DTT), beta-mercaptoethanol and glutathione.

16. The method of claim **9**, wherein the surfactant comprises one or more of sarcosyl and sodium dodecylsulfate.

17. The method of claim **9**, wherein the aggregation suppressor is selected from the group consisting of arginine, proline, polyethylene glycols, nonionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

18. The method of claim **9**, wherein the protein stabilizer comprises one or more of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes.

19. The method of claim **9**, wherein the redox component comprises one or more of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

20. The method of claim **9**, wherein the separation matrix is:

(i) an affinity resin, selected from the group consisting of Protein A, Protein G, and synthetic mimetic affinity resin; or

(ii) a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

21. The method of any one of claim **1** or **9-12**, wherein the protein is isolated after elution from the separation matrix.

22. The method of claim **8**, wherein the protein is isolated after refolding.

23. The method of claim **14**, wherein the reductant comprises one or more of cysteine, dithiothreitol (DTT), beta-mercaptoethanol and glutathione.

24. The method of claim **15**, wherein the surfactant comprises one or more of sarcosyl and sodium dodecylsulfate.

25. The method of claim **16**, wherein the aggregation suppressor is selected from the group consisting of arginine, proline, polyethylene glycols, nonionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

26. The method of claim **17**, wherein the protein stabilizer comprises one or more of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes.

27. The method of claim **18**, wherein the redox component comprises one or more of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

28. The method of claim **19**, wherein the separation matrix is:

(i) an affinity resin, selected from the group consisting of Protein A, Protein G, and synthetic mimetic affinity resin; or

(ii) a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

29. The method of claim **13**, wherein the protein is isolated after elution from the separation matrix.

30. The method of claim **20**, wherein the protein is isolated after elution from the separation matrix.

* * * * *

JS-CAND 44 (Rev. 06/17)

CIVIL COVER SHEET

The JS-CAND 44 civil cover sheet and the information contained herein neither replace nor supplement the filing and service of pleadings or other papers as required by law, except as provided by local rules of court. This form, approved in its original form by the Judicial Conference of the United States in September 1974, is required for the Clerk of Court to initiate the civil docket sheet. *(SEE INSTRUCTIONS ON NEXT PAGE OF THIS FORM.)*

I. (a) PLAINTIFFS
SANDOZ INC.

(b) County of Residence of First Listed Plaintiff *(EXCEPT IN U.S. PLAINTIFF CASES)*
Mercer County, NJ

(c) Attorneys *(Firm Name, Address, and Telephone Number)*
Erik J. Olson, Eric C. Pai; Morrison & Foerster LLP
755 Page Mill Road, Palo Alto, CA 94304 Tel: 650-813-5600

DEFENDANTS
AMGEN INC. and AMGEN MANUFACTURING, LIMITED

County of Residence of First Listed Defendant
(IN U.S. PLAINTIFF CASES ONLY) Ventura County, CA

NOTE: IN LAND CONDEMNATION CASES, USE THE LOCATION OF THE TRACT OF LAND INVOLVED.

Attorneys *(If Known)*

II. BASIS OF JURISDICTION *(Place an "X" in One Box Only)*

- ☐ 1 U.S. Government Plaintiff
- ☒ 3 Federal Question *(U.S. Government Not a Party)*
- ☐ 2 U.S. Government Defendant
- ☐ 4 Diversity *(Indicate Citizenship of Parties in Item III)*

III. CITIZENSHIP OF PRINCIPAL PARTIES *(Place an "X" in One Box for Plaintiff and One Box for Defendant)*
(For Diversity Cases Only)

	PTF	DEF		PTF	DEF
Citizen of This State	☐ 1	☐ 1	Incorporated *or* Principal Place of Business In This State	☐ 4	☐ 4
Citizen of Another State	☐ 2	☐ 2	Incorporated *and* Principal Place of Business In Another State	☐ 5	☐ 5
Citizen or Subject of a Foreign Country	☐ 3	☐ 3	Foreign Nation	☐ 6	☐ 6

IV. NATURE OF SUIT *(Place an "X" in One Box Only)*

CONTRACT
- ☐ 110 Insurance
- ☐ 120 Marine
- ☐ 130 Miller Act
- ☐ 140 Negotiable Instrument
- ☐ 150 Recovery of Overpayment Of Veteran's Benefits
- ☐ 151 Medicare Act
- ☐ 152 Recovery of Defaulted Student Loans (Excludes Veterans)
- ☐ 153 Recovery of Overpayment of Veteran's Benefits
- ☐ 160 Stockholders' Suits
- ☐ 190 Other Contract
- ☐ 195 Contract Product Liability
- ☐ 196 Franchise

REAL PROPERTY
- ☐ 210 Land Condemnation
- ☐ 220 Foreclosure
- ☐ 230 Rent Lease & Ejectment
- ☐ 240 Torts to Land
- ☐ 245 Tort Product Liability
- ☐ 290 All Other Real Property

TORTS

PERSONAL INJURY
- ☐ 310 Airplane
- ☐ 315 Airplane Product Liability
- ☐ 320 Assault, Libel & Slander
- ☐ 330 Federal Employers' Liability
- ☐ 340 Marine
- ☐ 345 Marine Product Liability
- ☐ 350 Motor Vehicle
- ☐ 355 Motor Vehicle Product Liability
- ☐ 360 Other Personal Injury
- ☐ 362 Personal Injury -Medical Malpractice

PERSONAL INJURY
- ☐ 365 Personal Injury – Product Liability
- ☐ 367 Health Care/ Pharmaceutical Personal Injury Product Liability
- ☐ 368 Asbestos Personal Injury Product Liability

PERSONAL PROPERTY
- ☐ 370 Other Fraud
- ☐ 371 Truth in Lending
- ☐ 380 Other Personal Property Damage
- ☐ 385 Property Damage Product Liability

CIVIL RIGHTS
- ☐ 440 Other Civil Rights
- ☐ 441 Voting
- ☐ 442 Employment
- ☐ 443 Housing/ Accommodations
- ☐ 445 Amer. w/Disabilities-Employment
- ☐ 446 Amer. w/Disabilities-Other
- ☐ 448 Education

PRISONER PETITIONS

HABEAS CORPUS:
- ☐ 463 Alien Detainee
- ☐ 510 Motions to Vacate Sentence
- ☐ 530 General
- ☐ 535 Death Penalty

OTHER:
- ☐ 540 Mandamus & Other
- ☐ 550 Civil Rights
- ☐ 555 Prison Condition
- ☐ 560 Civil Detainee - Conditions of Confinement

FORFEITURE/PENALTY
- ☐ 625 Drug Related Seizure of Property 21 USC 881
- ☐ 690 Other

LABOR
- ☐ 710 Fair Labor Standards Act
- ☐ 720 Labor/Management Relations
- ☐ 740 Railway Labor Act
- ☐ 751 Family and Medical Leave Act
- ☐ 790 Other Labor Litigation
- ☐ 791 Employee Retirement Income Security Act

IMMIGRATION
- ☐ 462 Naturalization Application
- ☐ 465 Other Immigration Actions

BANKRUPTCY
- ☐ 422 Appeal 28 USC § 158
- ☐ 423 Withdrawal 28 USC § 157

PROPERTY RIGHTS
- ☐ 820 Copyrights
- ☒ 830 Patent
- ☐ 835 Patent-Abbreviated New Drug Application
- ☐ 840 Trademark

SOCIAL SECURITY
- ☐ 861 HIA (1395ff)
- ☐ 862 Black Lung (923)
- ☐ 863 DIWC/DIWW (405(g))
- ☐ 864 SSID Title XVI
- ☐ 865 RSI (405(g))

FEDERAL TAX SUITS
- ☐ 870 Taxes (U.S. Plaintiff or Defendant)
- ☐ 871 IRS—Third Party 26 USC § 7609

OTHER STATUTES
- ☐ 375 False Claims Act
- ☐ 376 Qui Tam (31 USC § 3729(a))
- ☐ 400 State Reapportionment
- ☐ 410 Antitrust
- ☐ 430 Banks and Banking
- ☐ 450 Commerce
- ☐ 460 Deportation
- ☐ 470 Racketeer Influenced & Corrupt Organizations
- ☐ 480 Consumer Credit
- ☐ 490 Cable/Sat TV
- ☐ 850 Securities/Commodities/ Exchange
- ☐ 890 Other Statutory Actions
- ☐ 891 Agricultural Acts
- ☐ 893 Environmental Matters
- ☐ 895 Freedom of Information Act
- ☐ 896 Arbitration
- ☐ 899 Administrative Procedure Act/Review or Appeal of Agency Decision
- ☐ 950 Constitutionality of State Statutes

V. ORIGIN *(Place an "X" in One Box Only)*

- ☒ 1 Original Proceeding
- ☐ 2 Removed from State Court
- ☐ 3 Remanded from Appellate Court
- ☐ 4 Reinstated or Reopened
- ☐ 5 Transferred from Another District *(specify)*
- ☐ 6 Multidistrict Litigation-Transfer
- ☐ 8 Multidistrict Litigation-Direct File

VI. CAUSE OF ACTION

Cite the U.S. Civil Statute under which you are filing ***(Do not cite jurisdictional statutes unless diversity)***:
28 U.S.C. §§ 2201-2202; 35 U.S.C. § 1 et seq.

Brief description of cause:
Declaratory Judgment of Patent Noninfringement and Invalidity

VII. REQUESTED IN COMPLAINT:

☐ CHECK IF THIS IS A **CLASS ACTION** UNDER RULE 23, F.R.Cv.P.

DEMAND $

CHECK YES only if demanded in complaint:
JURY DEMAND: ☐ Yes ☒ No

VIII. RELATED CASE(S) IF ANY *(See instructions)*:

JUDGE Hon. Richard Seeborg DOCKET NUMBER 3:16-cv-02581-RS & 3:14-cv-04741-RS

IX. DIVISIONAL ASSIGNMENT (Civil Local Rule 3-2)

(Place an "X" in One Box Only) ☐ **SAN FRANCISCO/OAKLAND** ☐ **SAN JOSE** ☐ **EUREKA-MCKINLEYVILLE**

DATE February 21, 2019 **SIGNATURE OF ATTORNEY OF RECORD** /s/ Erik J. Olson

American LegalNet, Inc.
www.FormsWorkFlow.com

INSTRUCTIONS FOR ATTORNEYS COMPLETING CIVIL COVER SHEET FORM JS-CAND 44

Authority For Civil Cover Sheet. The JS-CAND 44 civil cover sheet and the information contained herein neither replaces nor supplements the filings and service of pleading or other papers as required by law, except as provided by local rules of court. This form, approved in its original form by the Judicial Conference of the United States in September 1974, is required for the Clerk of Court to initiate the civil docket sheet. Consequently, a civil cover sheet is submitted to the Clerk of Court for each civil complaint filed. The attorney filing a case should complete the form as follows:

I. a) Plaintiffs-Defendants. Enter names (last, first, middle initial) of plaintiff and defendant. If the plaintiff or defendant is a government agency, use only the full name or standard abbreviations. If the plaintiff or defendant is an official within a government agency, identify first the agency and then the official, giving both name and title.

b) County of Residence. For each civil case filed, except U.S. plaintiff cases, enter the name of the county where the first listed plaintiff resides at the time of filing. In U.S. plaintiff cases, enter the name of the county in which the first listed defendant resides at the time of filing. (NOTE: In land condemnation cases, the county of residence of the "defendant" is the location of the tract of land involved.)

c) Attorneys. Enter the firm name, address, telephone number, and attorney of record. If there are several attorneys, list them on an attachment, noting in this section "(see attachment)."

II. Jurisdiction. The basis of jurisdiction is set forth under Federal Rule of Civil Procedure 8(a), which requires that jurisdictions be shown in pleadings. Place an "X" in one of the boxes. If there is more than one basis of jurisdiction, precedence is given in the order shown below.

(1) United States plaintiff. Jurisdiction based on 28 USC §§ 1345 and 1348. Suits by agencies and officers of the United States are included here.

(2) United States defendant. When the plaintiff is suing the United States, its officers or agencies, place an "X" in this box.

(3) Federal question. This refers to suits under 28 USC § 1331, where jurisdiction arises under the Constitution of the United States, an amendment to the Constitution, an act of Congress or a treaty of the United States. In cases where the U.S. is a party, the U.S. plaintiff or defendant code takes precedence, and box 1 or 2 should be marked.

(4) Diversity of citizenship. This refers to suits under 28 USC § 1332, where parties are citizens of different states. When Box 4 is checked, the citizenship of the different parties must be checked. (See Section III below; **NOTE: federal question actions take precedence over diversity cases.**)

III. Residence (citizenship) of Principal Parties. This section of the JS-CAND 44 is to be completed if diversity of citizenship was indicated above. Mark this section for each principal party.

IV. Nature of Suit. Place an "X" in the appropriate box. If the nature of suit cannot be determined, be sure the cause of action, in Section VI below, is sufficient to enable the deputy clerk or the statistical clerk(s) in the Administrative Office to determine the nature of suit. If the cause fits more than one nature of suit, select the most definitive.

V. Origin. Place an "X" in one of the six boxes.

(1) Original Proceedings. Cases originating in the United States district courts.

(2) Removed from State Court. Proceedings initiated in state courts may be removed to the district courts under Title 28 USC § 1441. When the petition for removal is granted, check this box.

(3) Remanded from Appellate Court. Check this box for cases remanded to the district court for further action. Use the date of remand as the filing date.

(4) Reinstated or Reopened. Check this box for cases reinstated or reopened in the district court. Use the reopening date as the filing date.

(5) Transferred from Another District. For cases transferred under Title 28 USC § 1404(a). Do not use this for within district transfers or multidistrict litigation transfers.

(6) Multidistrict Litigation Transfer. Check this box when a multidistrict case is transferred into the district under authority of Title 28 USC § 1407. When this box is checked, do not check (5) above.

(8) Multidistrict Litigation Direct File. Check this box when a multidistrict litigation case is filed in the same district as the Master MDL docket.

Please note that there is no Origin Code 7. Origin Code 7 was used for historical records and is no longer relevant due to changes in statute.

VI. Cause of Action. Report the civil statute directly related to the cause of action and give a brief description of the cause. **Do not cite jurisdictional statutes unless diversity.** Example: U.S. Civil Statute: 47 USC § 553. Brief Description: Unauthorized reception of cable service.

VII. Requested in Complaint. Class Action. Place an "X" in this box if you are filing a class action under Federal Rule of Civil Procedure 23.

Demand. In this space enter the actual dollar amount being demanded or indicate other demand, such as a preliminary injunction.

Jury Demand. Check the appropriate box to indicate whether or not a jury is being demanded.

VIII. Related Cases. This section of the JS-CAND 44 is used to identify related pending cases, if any. If there are related pending cases, insert the docket numbers and the corresponding judge names for such cases.

IX. Divisional Assignment. If the Nature of Suit is under Property Rights or Prisoner Petitions or the matter is a Securities Class Action, leave this section blank. For all other cases, identify the divisional venue according to Civil Local Rule 3-2: "the county in which a substantial part of the events or omissions which give rise to the claim occurred or in which a substantial part of the property that is the subject of the action is situated."

Date and Attorney Signature. Date and sign the civil cover sheet.

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