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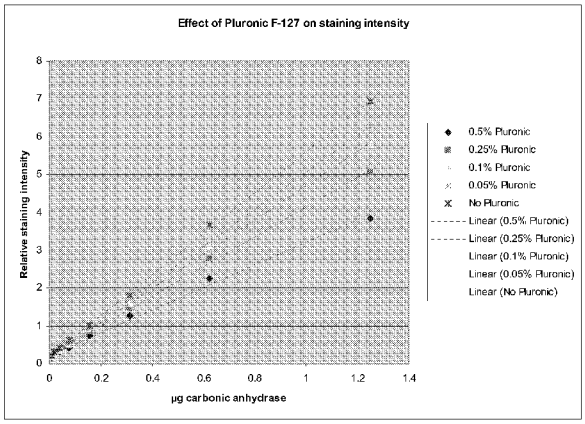
(54) Title: COLLOIDAL COOMASSIE STAIN

FIG. 1



(57) Abstract: Colloidal formulation for staining proteins and methods of their use are provided.

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COLLOIDAL COOMASSIE STAIN

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0001] The present application claims benefit of priority to US Provisional Patent Application No. 61/777,801, filed March 12, 2013, which is incorporated by referenced.

BACKGROUND OF THE INVENTION

[0002] The use of Coomassie Brilliant Blue G-250 for staining electrophoresis gels to a defined endpoint is possible because the dye can be formulated as a colloidal form that does not enter the gel for staining, but rather is adsorbed by the protein bands in the gel, saturating the bands given enough time to diffuse throughout the bands. The fact that the colloidal form does not enter the gel itself makes the background very low, and lowers the need for a destaining step, which can result in decreased intensity of the stained protein bands.

[0003] Typically, a two-part stain is used to stain electrophoesis gels. The two-part stain must be made fresh before each use to have the desired performance (e.g., low background). Two-part stains quickly form a precipitate that renders the solution unusable – typically in 24hrs. Two-part stain formulations are also based on using methanol as the alcohol. Simply replacing methanol with ethanol will render the formulation to have unacceptable performance.

BRIEF SUMMARY OF THE INVENTION

[0004] Stabilized colloid stain formulations for staining proteins in electrophoresis gels are provided.

[0005] In some embodiments, the formulation comprises: Coomassie Brilliant Blue; a surfactant; ethanol; ammonium sulfate; and optionally orthophosphoric acid. In some embodiments, the Coomassie Brilliant Blue is Coomassie Brilliant Blue G-250 or Coomassie Brilliant Blue R-250. In some embodiments, the surfactant is poloxamer 407. In some embodiments, the formulation comrpises 0.01-0.05% wt/wt Coomassie Brilliant Blue; 0.01-

0.10% wt/wt poloxamer 407; 10-20% wt/wt ethanol; 5-10% wt/wt ammonium sulfate; and 0.5-5% orthophosphoric acid.

[0006] In some embodiments, the formulation comprises a Coomassie Brilliant Blue dye; a surfactant; ethanol or methanol; and ammonium sulfate. In some embodiments, the formulation further comprises orthophosphoric acid. In some embodiments, the formulation comprises ethanol (and in some further embodiments does not comprise methanol). In some embodiments, the formulation comprises methanol (and in some further embodiments does not comprise ethanol).

[0007] In some embodiments, the Coomassie Brilliant Blue is depicted in Formula 1. In some embodiments, the Coomassie Brilliant Blue is depicted in Formula 2.

[0008] In some embodiments, the surfactant is a polyoxyethylene-containing surfactant. In some embodiments, the surfactant is a poloxamer surfactant. In some embodiments, the surfactant is poloxamer 407.

[0009] In some embodiments, the formulation comprises: 0.01-0.05% wt/wt Coomassie Brilliant Blue dye; 0.01-0.10% wt/wt a surfactant; 10-20% wt/wt ethanol; 5-10% wt/wt ammonium sulfate; and 0.5-5% orthophosphoric acid. In some embodiments, the surfactant is a polyoxyethylene-containing surfactant. In some embodiments, the surfactant is a poloxamer surfacant. In some embodiments, the surfactant is a poloxamer 407.

[0010] Also provided are methods of staining proteins in an electrophoresis gel using the formulations described herein. In some embodiments, the method comprises contacting the gel with a formulation of any of claims 1-10 as described herein for a sufficient time and under conditions to stain proteins in the gel, and detecting the presence or quantity of protein in at least one region of the gel. In some embodiments, the formulation is stored at least three days before the contacting.

[0011] Also provided are methods of storing a colloidal formulation for proteins in an electrophoresis gel. In some embodiments, the method comprises storing the colloidal formulation as described herein for at least 3 (e.g., at least 7, 10, 15, 30, or more) days.

[0012] Also provided is a kit comprising the colloidal formulation as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] FIG. 1 illustrates the effect of various (0.5%-0.05%) Pluronic F-127 concentrations in staining formulations on staining intensity.

[0014] FIG. 2 illustrates the effect of various lower (0.05% and below) Pluronic F-127 concentrations in staining formulations on staining intensity.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0015] The inventors have discovered that a stable colloidal Coomassie Briliant Blue stain can be generated by including a surfactant and ethanol in the formulation. The resulting colloidal formulation i) has a long shelf-life, ii) is environmentally attractive (low environmental impact formulation using ethanol), and iii) is easier to use than a two-part stain because fewer steps are required to use the formulation. The formulations described herein perform essentially identically to a traditional 2-part short shelf life colloidal Coomassie stain (e.g., as described in Neuhoff *et al.*, *ELECTROPHORESIS* 9(6):255–262 (1988).

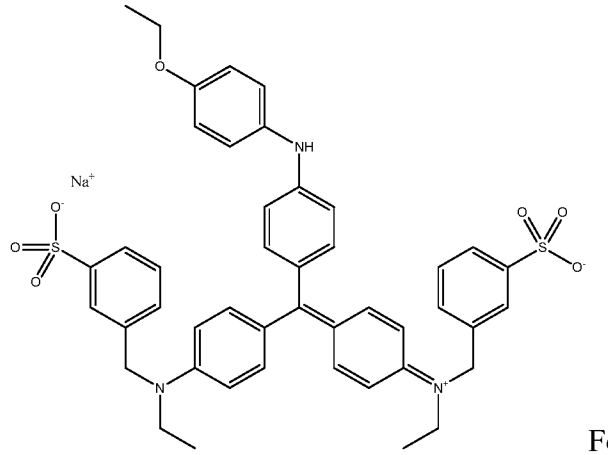
[0016] The formulations described herein have at least two notable aspects. First, ethanol is used as the alcohol. The quantity of ethanol, as well as of ammonium sulfate, can be optimized for the increased solubility of Coomassie in EtOH vs. MeOH, thereby allowing for a stable colloidal Coomassie formulation.

[0017] Second, a surfactant (e.g., Pluronic™ F-127 (generically known as poloxamer 407)) is added to stabilize the formulation and prevent precipitation, thereby allowing for a long shelf-stable 1-part solution.

II. Components for formulations

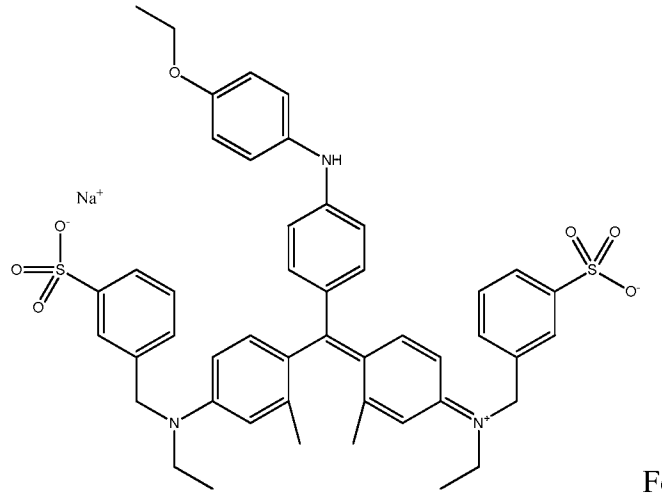
[0018] Colloidal dyes used for staining proteins can be used in the formulations described herein. Coomassie Brilliant Blue is a tradename for a class of dyes commonly used in protein staining. Examples include Coomassie Brilliant Blue G-250 and Coomassie Brilliant Blue R-

250. Coomassie Brilliant Blue R-250 is depicted below in Formula 1.



Formula 1.

[0019] Coomassie Brilliant Blue G-250 (CAS number 6104-58-1) differs from R-250 by inclusion of two methyl groups as showbn below in Formula 2.



Formula 2.

Generic terms for Coomassie Brilliant Blue R-250 (CAS number 6104-59-2) include Xylene Brilliant Cyanine G, as well as C.I. 42655, C.I. Acid Blue 90, Brilliant indocyanine G, and Brillantindocyanin G.

[0020] As noted above, the inclusion of a surfactant in the formulation provides for stability (e.g., lack of significant preciptation) of the formulation, allowing the formulation to be stored for long periods of time (e.g., based on accelerated shelf life studies, at least two years) prior to use. In view of the stability of the formulation, the formulation can be made as a "1-part" formulation (i.e., all staining ingredients in the same solution), thereby avoiding the step of preparing a fresh dye solution from a "2-part" formulation as is current practice in the industry. Without intending to limit the scope of possible surfactants that can be used in the formulations, in some embodiments, the surfactant is a polyoxypropylene-containing

surfactant such as a poloxamer surfactant. Poloxamer surfactants are characterized by a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)). Because the lengths of the polymer blocks can be customized, many different poloxamers exist that have slightly different properties. Poloxamer copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits, the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content (e.g., P407 = Poloxamer with a polyoxypropylene molecular mass of 4,000 g/mol and a 70% polyoxyethylene content). For the Pluronic and Synperonic poloxamer tradenames, coding of these copolymers starts with a letter to define its physical form at room temperature (L = liquid, P = paste, F = flake (solid)) followed by two or three digits, The first digit (two digits in a three-digit number) in the numerical designation, multiplied by 300, indicates the approximate molecular weight of the hydrophobe; and the last digit x 10 gives the percentage polyoxyethylene content (e.g., L61 indicates a polyoxypropylene molecular mass of 1,800 g/mol and a 10% polyoxyethylene content). In the example given, poloxamer 181 (P181) = Pluronic L61 and Synperonic PE/L 61. Exemplary poloxamer surfactants incude, but are not limited to, Pluronuics F-127, F-108, F-68, P-105, L-35, and P-123. In other embodiments, the surfactant is a polyoxyethylene-containing surfactant, i.e., a surfactant comprising one or more polyoxyethyl groups.

[0021] The formulations will comprise an alcohol such as ethanol or methanol. Historically, methanol has been used stain formulations because standard formulations with ethanol had unacceptable performance issues. However, as discussed herein, the inclusion of a surfactant allows for inclusion of ethanol instead of methanol. The inclusion of ethanol instead of methanol is preferred in many instances because of the environmental and personal dangers of methanol. Nevertheless, in some embodiments, the formulation can contain methanol.

[0022] The ionic strength and pH of the formulation determine the colloidal properties of the stain formulation. Ionic strength can be controlled by addition of an ionic species at a level to provide for the desired colloidal properties of the stain. An exemplary ionic species is ammonium sulfate. Similarly, a weak acid can be included in the formulation to adjust and maintain the pH of the formulation. Exemplary acids include, but are not limited to, orthophosphoric acid, acetic acid, trichlororacetic acid(TCA) and protocatechuic acid (PCA). Additional considerations for generation of optimal conditions of ionic strength and pH can

be found in, e.g., Neuhoff, *et al.*, *Electrophoresis* 6:427-448 (1985); Neuhoff *et al.*, *Electrophoresis* 11:101-117 (1990); and Neuhoff, *et al.*, *Electrophoresis* 9:255-262 (1988).

[0023] The concentration of ingredients in the colloidal formulation can vary within a range, but the formulation that shows the best enhancement of stability, while maintaining the sensitivity of an unstabilized (i.e., standard 2-part methanol-based) formulation, is about 10-20% (e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20%) by weight of ethanol, about 5-10% (e.g., 5, 6, 7, 8, 9, or 10, e.g., between 7% and 8%) by weight of ammonium sulfate, about 0.01-0.10% (e.g., 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, or 0.10%) by weight surfactant (e.g., poloxamer 407, e.g., sold by BASF under the name Pluronic™ F-127) or other surfactants as described above), 0.01-0.05% (e.g., 0.01%, 0.02%, 0.03, 0.04, or 0.05%) by weight Coomassie Brilliant Blue (it is believed either of Coomassie Brilliant Blue R-250 or Coomassie Brilliant Blue G-250 can be used), and approximately 0.5-5% (e.g., 0.5, 1, 2, 3, 4, or 5%) by weight orthophosphoric acid. The formulation will be formulated in water, e.g., deionized water.

III. Methods

[0024] Any gel in which proteins have been electrophoresed can be stained with the formulations described herein according to standard staining protocols (*e.g.*, by methods described in Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or *Current Protocols in Molecular Biology, Volumes 1-3*, John Wiley & Sons, Inc. (1994-1998)). Examples of gels include, e.g., polyacrylamide and agarose gels. The formulations can also be used for gels in which the proteins have been separated by, e.g., isoelectric focusing and for 2-D gels. The matrix may also be a porous particle. Additionally, the invention can be used to stain proteins on and/or in membranes or filters made from natural or artificial materials such as cellulose or derivatized versions thereof (e.g., nitrocellulose) and nylon or derivatized versions thereof (e.g., PVDF). The protein may also be applied to the solid matrix or support by capillary action or wicking, chromatography, electrophoresis or electrofocussing, or other methods such as, for example, western blotting and immunoblotting.

EXAMPLE

[0025] A series of formulations were generated to make a stain that was safer and more stable than current methanol-based 2-part protein stains. An exemplary standard 2-part

staining formulation comprised 8% ammonium sulfate, 1.6% orthophosphoric acid, 0.08% Coomassie Brilliant Blue G-250, and 20% methanol, formulated in deionized water. Initial attempts too simply replace the methanol with ethanol failed, producing a formulation with significant precipitates.

[0026] Subsequently, the surfactant Pluronic F-127 was added to a formulation of Coomassie Brilliant Blue G-250 containing 7.8% ammonium sulfate, 2.2 % orthophosphoric acid, and 14.2% ethanol. The formulation was prepared with various levels of Pluronic F-127 and the stain was stored in a covered dish for 5 days. Material was poured off and the remaining precipitate was visualized. Concentrations as low as 0.05% Pluronic F-127 prevented precipitates from forming. Further, the effect of surfactant concentration on relative protein staining was tested. The results are shown in FIG. 1-2 and show that the concentration of surfactant only had a minimal effect on protein (carbonic anhydrase) staining, especially at concentrations of Pluronic F-127 at or below 0.005% (Figure 2).

[0027] An accelerated shelf life assay was performed with the formulation having 0.05% Pluronic F-127. The formulation was stored for 30 weeks at 37° C, and a second batch of the formulation was stored for 11 weeks at 50° C. Each of these stored formulations was then used to stain a protein polyacrylamide gel and generated acceptable results, i.e., equivalent to staining results of a freshly-made 2-part methanol-based stain.

[0028] In the claims appended hereto, the term "a" or "an" is intended to mean "one or more." The term "comprise" and variations thereof such as "comprises" and "comprising," when preceding the recitation of a step or an element, are intended to mean that the addition of further steps or elements is optional and not excluded. All patents, patent applications, and other published reference materials cited in this specification are hereby incorporated herein by reference in their entirety. Any discrepancy between any reference material cited herein or any prior art in general and an explicit teaching of this specification is intended to be resolved in favor of the teaching in this specification. This includes any discrepancy between an art-understood definition of a word or phrase and a definition explicitly provided in this specification of the same word or phrase.

WHAT IS CLAIMED IS:

1. A colloidal formulation for staining proteins in electrophoresis gels, the formulation comprising

a Coomassie Brilliant Blue dye;

a surfactant;

ethanol or methanol;

ammonium sulfate.

2. The colloidal formulation of claim 1, further comprising orthophosphoric acid.

3. The colloidal formulation of claim 1, wherein the formulation comprises ethanol.

4. The colloidal formulation of claim 3, wherein the formulation does not comprise methanol

5. The colloidal formulation of claim 1, wherein the formulation comprises methanol and does not comprise ethanol.

6. The colloidal formulation of claim 1, wherein the Coomassie Brilliant Blue is depicted in Formula 1.

7. The colloidal formulation of claim 1, wherein the Coomassie Brilliant Blue is depicted in Formula 2.

8. The colloidal formulation of any of claims 1-7, wherein the surfactant is a polyoxyethylene-containing surfactant.

9. The colloidal formulation of any of claims 1-7, wherein the surfactant is a poloxamer surfactant.

10. The colloidal formulation of claim 1 or 6, wherein the surfactant is poloxamer 407.

11. The colloidal formulation of claim 1, comprising:

0.01-0.05% wt/wt Coomassie Brilliant Blue;
0.01-0.10% wt/wt a surfactant;
10-20% wt/wt ethanol;
5-10% wt/wt ammonium sulfate; and
0.5-5% orthophosphoric acid.

12. The colloidal formulation of claim 11, wherein the surfactant is a polyoxyethylene-containing surfactant.

13. The colloidal formulation of claim 11, wherein the surfactant is a poloxamer surfacant.

14. The colloidal formulation of claim 13, wherein the surfactant is a poloxamer 407.

15. A method of staining proteins in an electrophoresis gel, the method comprising,
contacting the gel with the formulation of any of claims 1-14 for a sufficient time and under conditions to stain proteins in the gel, and
detecting the presence or quantity of protein in at least one region of the gel.

16 . The method of claim 15, wherein the formulation is stored at least three days before the contacting.

17. A method of storing a colloidal formulation for proteins in an electrophoresis gel, the method comprising,
storing the colloidal formulation of any of claims 1-14 for at least 3 (e.g., at least 7, 10, 15, 30, or more) days.

18. A kit comprising the colloidal formulation of any of claims 1-14.

FIG. 1

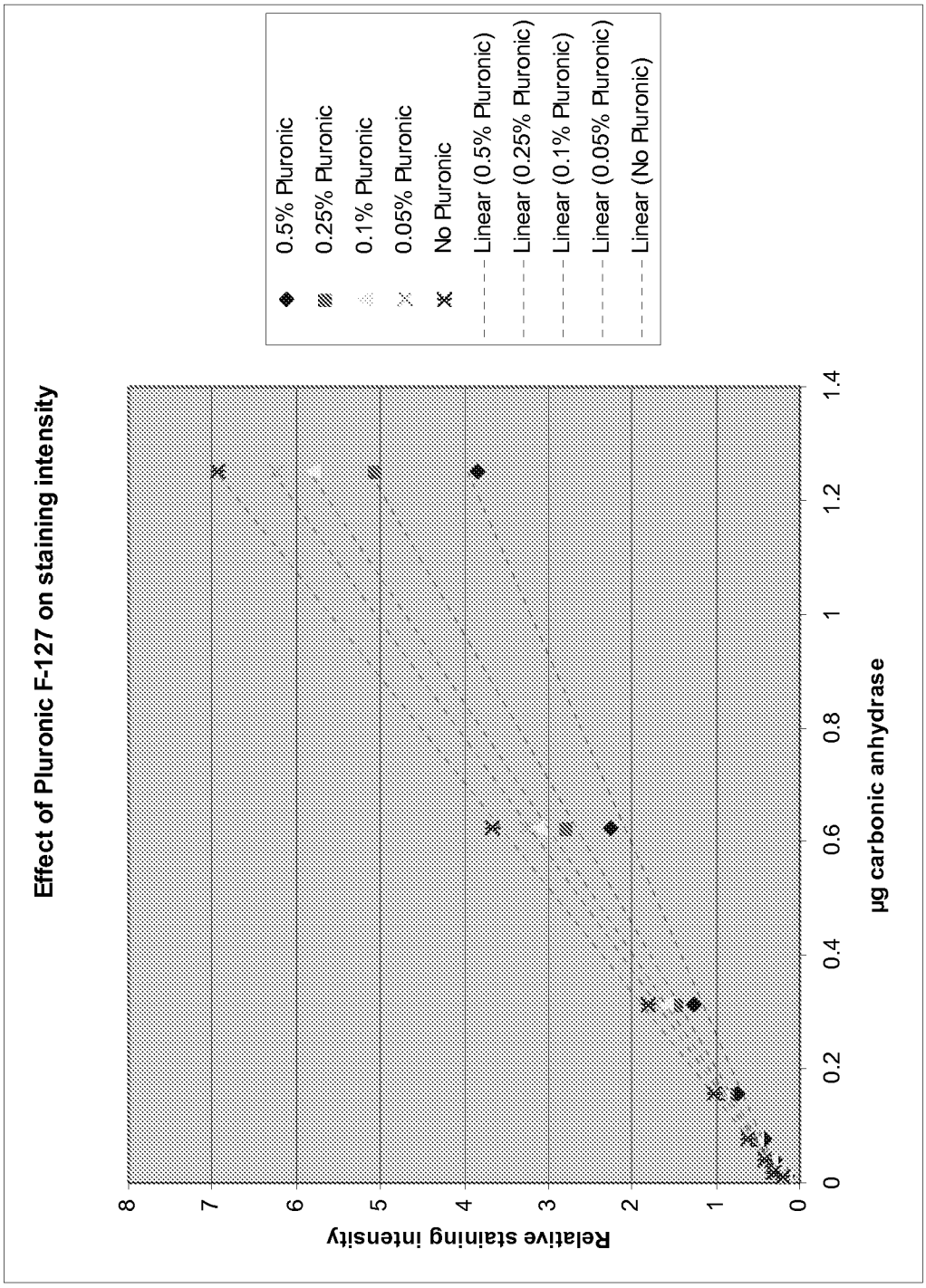
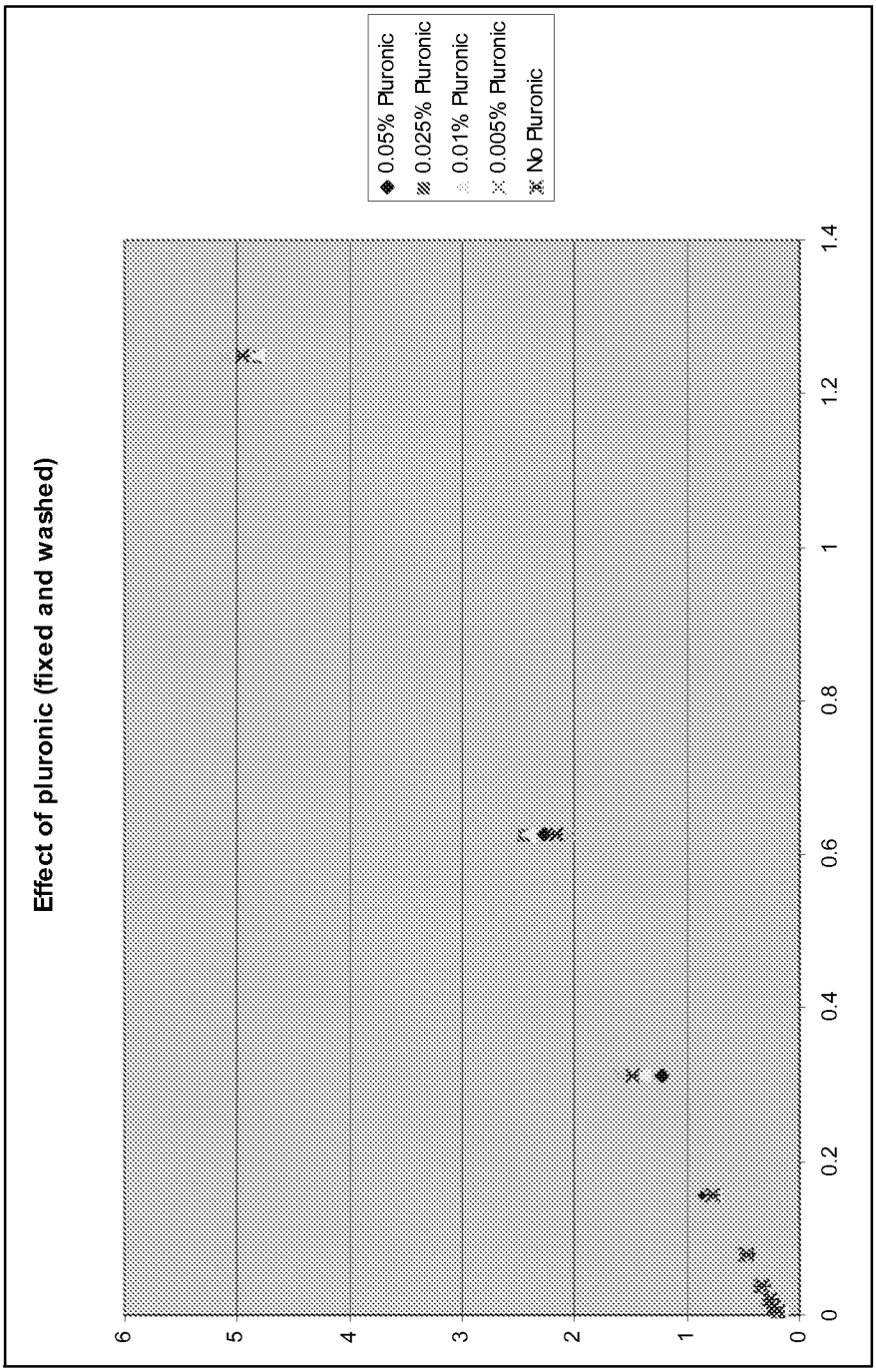

Effect of Pluronic F-127 on staining intensity
Relative staining intensity
µg carbonic anhydrase
0
1
2
3
4
5
6
7
8
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0.5% Pluronic
0.25% Pluronic
0.1% Pluronic
0.05% Pluronic
No Pluronic
Linear (0.5% Pluronic)
Linear (0.25% Pluronic)
Linear (0.1% Pluronic)
Linear (0.05% Pluronic)
Linear (No Pluronic)

FIG. 2


Effect of pluronic (fixed and washed)
0.05% Pluronic
0.025% Pluronic
0.01% Pluronic
0.005% Pluronic
No Pluronic
Relative staining intensity
µg carbonic anhydrase
0
1
2
3
4
5
6
0
0.2
0.4
0.6
0.8
1
1.2
1.4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/23520

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 1/30, 27/447, 33/68 (2014.01)
USPC - 436/86; 204/456, 462
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): G01N 1/30, 27/447, 33/68 (2014.01)
USPC: 436/86; 204/456, 462

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google Scholar; Google; ProQuest; coomassie brilliant blue (CBB), electrophoresis, protein staining, surfactants, poloxamer, ethanol, methanol, ammonium sulfate, phosphoric acid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4239495 A (GINDLER, EM et al.) December 16, 1980; column 2, lines 2-10; column 2, lines 18-37; column 2, lines 55-66	1-7, 8/1-7, 9/1-7, 10/1, 10/6, 11-14
Y	NEUHOFF, V et al. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis. 1988. Vol. 9. pages 255-262; page 256, left column; abstract	1-7, 8/1-7, 9/1-7, 10/1, 10/6, 11-14
A	US 4023933 A (BRADFORD, MM et al.) May 17, 1977; entire document	1-7, 8/1-7, 9/1-7, 10/1, 10/6, 11-14

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search	Date of mailing of the international search report
23 May 2014 (23.05.2014)	06 JUN 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/23520

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 15-18
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.