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(54) Title: CHEMILUMINESCENT ASSAY FOR dsDNA ANTIBODIES

(57) Abstract

An assay for systemic lupus erythematosus based upon capture of the anti-dsDNA portion of IgG in a human serum specimen by the Fc part of a molecule using solid phase immobilized F(ab')2 fragment of anti-human IgG specific for Fc, the captured IgG being then incubated with a synthetic dsDNA tagged with a moiety from which a signal proportional to the quantity of said synthetic dsDNA can be elicited. Upon eliciting a signal from the moiety, the amount of antibody to dsDNA can be quantified, providing diagnostic and prognostic information regarding the disease.

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CHEMILUMINESCENT ASSAY FOR dsDNA ANTIBODIES

This invention relates to biochemical assays, and more particularly to assays for antibodies to double-stranded nucleic acids.

Polymeric chains of deoxyribonucleic acid (DNA), being integral parts of the nuclear material of biological cells in double-stranded form (dsDNA), do not ordinarily stimulate the immune system to form antibodies. At least one disease, systemic lupus erythematosus, is however characterized by apparent abnormalities of the immune system that causes antibodies to be formed to dsDNA with fatal results. Antibodies to dsDNA occur almost exclusively in systemic lupus erythematosus and assay for such antibodies is one of the laboratory tests used in the diagnosis and determination of the prognosis of the disease.

Heretofore, measurement of anti-dsDNA antibody has typically been accomplished by using a radioactive immunoassay in which a complex formed between the antibody and antigen is labelled with a radioactive material, and a measurement of the radioactivity of the resulting product is measured as with a Geiger counter, photochemically or the like. The radioactive materials used in such assays may pose a significant danger, and where they have limited half-lives, frequent calibration is necessary. Alternatively one can use an enzyme-linked immunoabsorbent assay employing an enzyme linked to a molecule specific to an antibody bound to an antigen on a solid phase, e.g. peroxidase phosphate. A chromogenic substrate is added to generate a colored end product in the presence of the enzyme portion of the ligand. While such an assay is stable, it is far less sensitive than radioimmunoassays. Unfortunately also, rheumatoid factors tend to react with the Fc portion of the human IgG and thus may seriously interfere with the assay.

The known Crithidia assay does assay for true dsDNA but, being at best semi-quantitative, cannot follow the activity of the disease.

A principal object of the present invention is therefore to provide an assay for anti-dsDNA antibody, which assay is stable, yet highly sensitive, and uses no materials that pose a significant health threat to the assayer. Yet other objects of the present invention are to provide such an assay that, being linearly quantitative, has particular utility in clinical medicine; and to provide such an assay that measures only IgG antibody that relates to the disease activity.

1 Generally these and other objects of the present invention are realized by an assay
2 that incorporates a capture system for double stranded IgG antibody and thereby allows
3 measurement of the antibody only. The assay captures the anti-dsDNA portion of IgG in a
4 human serum specimen by the Fc part of a molecule using solid phase immobilized F(ab')2
5 fragment of anti-human IgG specific for Fc, the captured IgG being then incubated with a
6 synthetic dsDNA tagged with a moiety from which a signal proportional to the quantity of
7 said synthetic dsDNA can be elicited. Upon eliciting a signal from the moiety, one may
8 quantify the amount of antibody to dsDNA.
9 More specifically, a preferred embodiment of the present invention involves
10 immobilizing F(ab')2 fragment of anti-human IgG antibody, such as goat anti-human IgG
11 antibody, specific for the Fc part of a molecule, on an inert substrate or immunoadsorbent.
12 The present invention employs F(ab')2 to avoid rheumatoid factors that would otherwise
13 interfere with the assay because the latter tend to react with the Fc portion of the IgG. A
14 dilution of a human patient serum specimen believed to contain an anti-dsDNA portion of
15 IgG, is then permitted to contact the coated substrate under conditions and for a period of
16 time sufficient for a proportion of the patient IgG to become bound by reaction with the
17 animal anti-IgG antibody. Synthetic double-stranded DNA is labelled, for example, with
18 digoxigenin inasmuch as the use of the latter must result in dsDNA antibody that does not
19 have a single-stranded or denatured component. The digoxigenin is added to the serum,
20 again under conditions and for a time sufficient to bind to any anti-DNA antibodies which
21 may be present in the serum. The extent to which the labelled dsDNA is bound to the solid
22 phase immunoadsorbent is determined by adding an anti-digoxigenin antibody coupled to
23 alkaline phosphatase. The amount of alkaline phosphatase present is measured by the further
24 addition of a chemiluminescent substrate and the resulting luminescence due to reaction of
25 the alkaline phosphatase with the substrate, if any, is measured with a luminometer. The use
26 of chemiluminescence as a tag or label provides sufficient sensitivity to provide excellent
27 quantitative determination. The levels of luminescence obtained are compared to standard
28 levels provided by the similar reaction of standard quantities of anti-dsDNA antibodies and
29 expressed as U/mL, thereby relating the amount of bound labelled synthetic double-stranded
30 DNA detected to a predetermined quantitative relationship between the amount of labelled
31 double-stranded DNA and the amount of animal anti-human IgG antibody to determine the
32 amount of human IgG in the serum.

1 Pure double-stranded DNA molecules are provided for use in the anti-dsDNA assay.
2 The synthesis of the DNA from a single-stranded DNA M13 phage molecule is initiated with
3 random primers and carried out with dATP, dGTP, dCTP, dTTP and digoxigenin-labelled
4 deoxyuridine-triphosphate in the presence of sequenase enzyme, as will be described
5 hereinafter in detail.
6 Not all antibodies present in individuals have the same affinity or avidity for the
7 dsDNA antigen as does the standard antibody preparation. Differences in avidity between
8 the standard and the individual patient samples results in non-linear dose response curves.
9 Because of this non-linearity, a two-fold dilution of a sample may not result in a U/mL value
10 that is one-half of the undiluted sample result. The binding activity, however, of a patient
11 sample will be reproducible and consistent at a given dilution. For this reason, all samples
12 with u/mL over a 150 U standard are diluted as little as possible for reanalysis, and some
13 patients may need to always be analyzed at a set dilution so that sequential results over time
14 can be compared. Molecular biology techniques are used
15 to generate pure, double-stranded DNA molecules for use in the anti-dsDNA assay. The
16 DNA is typically synthesized from single-stranded DNA M13 circular phage molecules (0.2
17 μg/μL, USB #70704). This DNA synthesis is initiated with random primers and carried out
18 with dATP, dGTP, dCTP, dTTP and digoxigenin-labeled deoxyuridine-triphosphate (duTP)
19 (Boeringer Mannheim #1093088), in the presence of sequenase enzyme, a DNA polymerase
20 (USB #707775). Digoxigenin is a known steroid that occurs naturally in digitalis plants.
21 · All pipet tips used for this procedure must be autoclaved and should not be touched
22 without gloved hands.
23 An example of the DNA labeling/synthesis procedure is as follows: An M13
24 sequencing forward primer (24 Mer), 20μM (New England Biolabs #1224), is annealed to
25 the M13 DNA by pipetting 30 μL of 20μM forward primer and 120 μL of 5X sequenase
26 buffer (USB #70765) into a Perkin Elmer reaction tube. A mixture is formed by pipetting
27 5 μL of the annealed M13 DNA and 10 μL of the single-stranded DNA M13 circular phage
28 molecules (0.2 μg/μL) into a reaction tube where they are mixed, spun in a centrifuge for
29 about five seconds or less, and placed into a Perkin-Elmer DNA Thermal Cycler machine.
30 The mixture is heated in the machine to 65°C over two minutes, such temperature held for
31 five minutes, and then allowed to cool slowly to about 4°C over fifteen minutes. When
32 cooled, the tube is removed from the machine and placed immediately on ice. While the

1 primer is annealing, a labeling cocktail mixture is prepared by adding 5.50 μL each of 10μM
2 adenine stock solution, guanine stock solution, and cytosine stock solution, 3.575 μL of
3 10μM thymidine stock solution, 19.250 μL of 1 μM digoxigenin labeled duTP stock solution,
4 27.50 μL of 0.1M dithiothreitol, (USB (#70726), and 15.675 μL of distilled water, to a total
5 of 82.50μL.
6 To an empty reaction tube, 56 μL of the enzyme dilution buffer is added. The
7 sequenase enzyme is removed from the freezer where it should be kept, 8 μL thereof is added
8 to the enzyme dilution buffer, and 55 μL of this mixture is added to the labelling cocktail
9 mixture. 5 μL of this prepared labeling cocktail/sequenase solution is added to the iced tube
10 containing M13 DNA with annealed primer, mixed well and pulse spun. The tube is placed
11 into a "floater" in a 37°C water bath, incubated for 45 minutes, removed and placed on ice
12 and immediately 2 μL 0.5M EDTA is added to stop any further reaction. A kit for labeling
13 reactions of 10ng-3μg DNA with digoxigenin-labeled deoxyuridine-triphosphate is
14 commercially available from Boehringer-Mannheim Chemicals, Indianapolis, Indiana,
15 together with instructions for carrying out the procedure.
16 Known agarose gel electrophoresis and modified Southern blot procedures are
17 preferably used to check that the molecular weight of the newly prepare DNA and the
18 efficiency of the digoxigenin labeling are correct. Ultraviolet spectrophotometry is also
19 preferably employed to adjust the concentration of the DNA to lots previously prepared and
20 to ensure its purity.
21 The present invention preferably involves the preparation of an appropriate solid-
22 phase support for anti-human IgG antibody. First an 0.05 M Tris buffer, ph 9.5 is made by
23 weighing out 5.789 gm Tris base (Sigma Chemical Co., St. Louis. Mo., T-1503) and 0.3346
24 gm Tris acid (Sigma, T-3253). These materials are added to a 1.0 L graduated cylinder,
25 Q.S. to 1.0 L with distilled water and the pH checked to ensure that it is at 9.5. This buffer
26 is stored at 4°C where it will remain stable for about three months. Preferably the day before
27 the assay is to be performed, a 2.5 uG/mL solution of the capture antibody, preferably an
28 animal anti-human IgG antibody, such as goat anti-human IgG (Fc specific Jackson #109-006-
29 008 supplied at 1.1 mg/mL), is prepared by adding 25 μL of the stock F(ab)'2 anti-human
30 IgG (1.1 mg/mL to 11 mL of the 0.05 M Tris coating buffer (1:500 dilution). Using a
31 Titerteck 8 channel pipet, 100 μL of the anti-IgG/coating buffer is added to each well of a
32 96-well black, microfluor, microtiter plate. The plate is covered with a plastic plate sealer

1 and incubated for 6 hours at room temperature.

2 The hybridization procedure to carry out the assay of the present invention is
3 described in a document entitled, Genius™, Nonradioactive DNA Labeling and Detection Kit,
4 published by Boehringer-Mannheim, September, 1988, the contents of which are incorporated
5 herein by reference.

6 Specifically, a blocking buffer is prepared by adding 250 mg bovine serum albumin
7 (Sigma A-2153) to 25 mL of the 0.05 M Tris buffer and mixing gently to ensure complete
8 solubilization of the bovine serum albumin in the buffer. The coating solution is now flicked
9 out of the microtiter plate into a sink, the plate is inverted and the tops of the wells blotted
10 with paper towels to dry thoroughly each well. Then, 200 μL of blocking buffer is added
11 to each well, the plate is covered with a plastic plate sealer and incubated at 4°C overnight.

12 Preparatory to performing the assay of the present invention, fresh solutions of
13 washing and sample dilution buffers are prepared by as follows.

14 A 0.01M phosphate buffer solution -10X (hereinafter PBS) is prepared by weighing
15 out the following:

16 85.0 g. NaCl (Sigma, S-9625)
17 11.5 g. Na_2HPO_4 (Baker Chemical Co., Phillipsburg, N.J. 3828-5
18 anhydrous)
19 2.0 g. KCl (Sigma, P-4504)
20 2.0 g. KH_2PO_4 (Sigma, P-5379)
21 1.0 g. Thimerosal (Sigma, T-5125)

22 The foregoing materials are placed in a 1.0L graduated cylinder, distilled water is
23 added to Q.S. to 1.0L, and the solution mixed. This stock solution of phosphate buffer
24 solution (PBS) should be at pH 6.8.

25 A PBS/TWEEN buffer (0.01M PBS/0.05% polyoxyethylene-sorbitan monolaureate,
26 (hereinafter Tween)) buffer is prepared by mixing 100 mL of 10X PBS solution with 900 mL
27 of distilled water. 0.5 mL of Tween (Sigma, P-1379) is added and mixed gently to avoid
28 foaming.

29 A PBS wash buffer (0.01M PBS) is prepared by mixing 45 mL of stock 10X PBS
30 solution with 450 mL of distilled water.

31 A sample dilution buffer (0.01M PBS/ 0.05% Tween/ 0.02% bovine serum albumin
32 (hereinafter BSA)) is prepared by mixing 5.0 mL with 45 mL of distilled water and then

1 adding 50 μL of Tween and 10 mg BSA (Sigma, A-2135).
2 Standards and controls are prepared as follows from a stock serum sample taken from
3 a patient with a known high concentration of DNA antibodies:
4 A "high" standard is prepared from by dilution in diluting buffer to a standardized
5 IgG concentration of 825 mg/mL, as by mixing an appropriate amounts of μL of the standard
6 or stock serum in the diluting buffer.
7 A "medium" standard, prepared from the "high" standard to be 60% of the value
8 of the latter, is made by mixing 600 μL of high standard with 400 μL of diluting buffer.
9 Dilutions are prepared for all controls and standards by adding 10 μL of serum of
10 each to 490 μL of sample dilution buffer (BSA) in respective test tubes. For patients from
11 a previous run which proved to be strongly positive above the highest standard value, two
12 dilutions are made, one being a mixture of 50 μL aliquots of normal Lit serum and the
13 patient serum, the other being a mixture of 150 μL normal Lit serum and 50 μL of the
14 patient serum. To two 490 μL aliquots of the sample dilution buffer, 10 μL are added from
15 each of these dilutions to provide 1:50 dilutions. Similarly, 1:50 dilutions are prepared for
16 each patient sample to be assayed.
17 The incubated plate, prepared as hereinbefore described is placed on a platewasher
18 and the blocking buffer is aspirated from it. The aspirated plate is then washed with
19 PBS/Tween buffer a total of six times. The plate is then removed from the washer, blotted
20 with paper towels so that the wells are completely free of buffer. For each standard, control
21 and patient sample, 100 μL of each is added to respective wells in the plate, and 100 μL of
22 the sample dilution buffer is added to duplicate blank wells. The plate is then covered with
23 a plate sealer and incubated in a humid chamber at 37°C for 1 1/2 hours. Just prior to the
24 end of the incubation period a 1:500 dilution is made of the dsDNA-digoxigenin solution by
25 adding 24 μL of stock DNA solution to 12 mL of diluting buffer (0.1 μL/mL) At the end
26 of the incubation period, the diluted sample solution are aspirated and the plate is washed
27 with PBS/Tween buffer. The plate is then blotted and tapped to dry completely.
28 With a channel pipet, 100 μL aliquots of the diluted dsDNA-digoxigenin solution are
29 added to each well of the plate which is then covered with a plate sealer and again incubated
30 in a humid chamber at 37°C for 1 1/2 hours. At the end of this incubation time, the diluted
31 dsDNA-digoxigenin solution is aspirated from the plate and the plate washed with
32 PBS/Tween buffer. The plate is then blotted and tapped to dry completely. Just before the

1 end of the incubation period, a 1:500 dilution is made of the stock anti-digoxigenin solution
2 in sample diluent, and 100 μL aliquots of this dilution are added to each well. Again the
3 plate is covered with a plate sealer and again incubated for a third time in a humid chamber
4 at 37°C for 1 1/2 hours. At the end of this third incubation, the diluted stock anti-
5 digoxigenin solution is aspirated from the plate, the plate washed with PBS/Tween buffer and
6 the latter replace then with PBS without Tween. The wash lines are primed to ensure
7 complete removal of the PBS/Tween buffer, the plate washed with PBS without Tween,
8 blotted and tapped to dry completely.

9 During the third incubation period at least 12 mL of the chemiluminescence alkaline
10 phosphatase substrate, such as Lumiphos 530, is removed from refrigeration, allowed to
11 come to room temperature, and 100 μL aliquots thereof added to each dried well after the
12 incubation period. Detection of the digoxigenin-labeled probe is preferably accomplished
13 with an antibody-enzyme conjugate such as anti-digoxigenin-alkaline phosphatase. The latter
14 may be visualized by known enzyme-linked color reactions but in the present invention, is
15 preferably accomplished by chemiluminescent detection. To this end, the support carrying
16 the hybridized probe and bound antibody conjugate, is reacted with a known
17 chemiluminescent substrate for alkaline phosphatase, such as a 1,2-dioxetane commercially
18 available in a solution known as Lumi-Phos™ 530 from Boehringer Mannheim. The reaction
19 protocol can be carried out as described in a publication entitled Lumi-Phos™ 530 available
20 from Lumigen, Inc., a division of Boehringer Mannheim Chemicals. In order to detect the
21 chemiluminescence, it is preferred to use a luminometer such as an Amerlite™
22 chemiluminescent detector available from Eastman Kodak Co., Rochester, New York.

23 Immediately following the addition of the chemiluminescent substrate, the plate is
24 placed into a luminometer and run according to the instructions for that particular device,
25 providing readings for each well. The plate is left in the machine and at the end of a 45
26 minute incubation period, the machine instructed to reread the wells, providing a second set
27 of data. To determine if the reaction is at an endpoint, the initial and final readings of the
28 "medium" and "high" standards are compared and should be within 5 units for each sample.
29 Preferably, the machine will provide curve fitting statistics and the mean value for each set
30 of replicate wells for all standards, controls and patients. Normal individuals with have 15
31 U/mL of dsDNA antibody.

32 A run is acceptable on the Amerlite detector if all of the following criteria are met:

(1) the curve fit factor is <10.0; the light index is >10.0; and the % difference for each point in the standard curve provided by the luminometer is <5.0, (2) for the controls, the "medium" and "high" controls at the beginning and end of the batch of samples are each within 5 units; replicate wells should have CVs less than 10%; and the mean values of all controls must be within their accepted duality control limits, and (3) for the patient samples, replicate wells should have CVs less than 10%; and values above the highest standard must be repeated on a subsequent run to obtain a value within the standard curve.

For patients that are run in dilutions of normal serum, the values may be calculated as follows:

For the 1:2 volume dilution:.

Reported value in U/mL = A x (B+C)/B

where A = U/mL from the standard curve ;

B = IgG level in the patient serum; and

C = IgG level in the normal control serum.

For the 1:4 volume dilution:

Reported value = A + (B+3C)/B

For all patient samples above the top standard, the next run should be repeated with additional dilutions. It will be appreciated that readings above the top standard are diagnostic of a diseased condition characterized by antibodies formed to dsDNA, most probably systemic lupus erythematosus. It is suggested that as such disease progresses, the extent to which the assay increases beyond the normal U/ml of dsDNA antibody is indicative of the activity of the disease.

Since certain changes may be made in the above method without departing from the scope of the invention herein involved, it is intended that all matter contained in the above description or shown in the accompanying drawings shall be interpreted in an illustrative and not in a limiting sense.

What is claimed:

1 1. A method of assaying for antibodies to human double-stranded DNA in a liquid,
2 human-serum specimen, said method comprising the steps of:
3 forming a first incubation mixture of said specimen and a solid phase
4 immunoadsorbent having immobilized thereon animal anti-human IgG antibody including
5 F(ab')2 fragment specific for Fc;
6 incubating said first mixture under conditions and for a period of time sufficient for
7 Fc in human IgG in said specimen to become bound by said F(ab')2 fragment in said animal
8 anti-IgG antibody;
9 forming a second incubation mixture of said first mixture and digoxigenin-labelled
10 synthetic double-stranded DNA under such conditions and for a period of time sufficient to
11 bind to said immunoadsorbent any anti-DNA antibodies which may be present in the serum
12 so as to form a double-stranded DNA antibody that has substantially no single stranded or
13 denatured components;
14 detecting the amount of digoxigenin labelled synthetic double-stranded DNA bound
15 to said immunoadsorbent; and
16 relating the amount of bound labelled synthetic double-stranded DNA detected to a
17 predetermined quantitative relationship between the amount of labelled double-stranded DNA
18 and the amount of animal anti-human IgG antibody to determine the amount of human IgG
19 in said specimen.

1 2. A method of assaying for antibodies to human double-stranded DNA (dsDNA)
2 in a human serum specimen, said method comprising the steps of:
3 capturing the anti-dsDNA portion of IgG in said human serum specimen by the Fc
4 part of a molecule using solid phase immobilized F(ab')2 fragment of anti-human IgG specific
5 for Fc;
6 incubating the captured IgG with synthetic dsDNA tagged with a moiety from which
7 a signal proportional to the quantity of said synthetic dsDNA can be elicited;
8 eliciting said signal; and
9 quantifying the amount of antibody to dsDNA in accordance with the elicited signal.

1 3. A method as defined in claim 2 wherein said
2 captured IgG is incubated with synthetic dsDNA tagged with alkaline phosphatase,
3 and said elicited signal is proportional to the quantity of alkaline phosphatase tagging said
4 synthetic dsDNA.

1 4. A method as defined in claim 3 including the step of incubating comprises:
2 adding chemiluminescent substrate to said antibody, and
3 measuring any resulting luminescence from the reaction of said substrate with said
4 phosphatase.

1 5. A method as defined in claim 2 wherein said step of capturing is effected by:
2 coating animal anti-human IgG antibody onto an inert substrate;
3 contacting the coated substrate with a dilution of said serum so that a proportion of the
4 patient IgG becomes bound by reaction with said animal anti-IgG antibody; and
5 labelling synthetic double-stranded DNA with digoxigenin and is added the labelled
6 DNA to said serum to bind to any anti-DNA antibodies which may be present in the serum.

1 6. A method as defined in claim 5 including the step of incubating comprises:
2 contacting said labelled DNA with an anti-digoxigenin antibody coupled to alkaline
3 phosphatase,
4 adding a chemiluminescent substrate to said anti-digoxigenin antibody, and
5 measuring any resulting luminescence from the reaction of said substrate with said
6 phosphatase.

1 7. A method as defined in claim 5 including the further step of comparing the
2 measurement of said luminescence with predetermined standard levels.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/00, 33/53; C12Q 1/68
US CL :435/6; 435/7.1, 436/508

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)

U.S. : 530/387.1, 435/6; 435/7.1, 436/508

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)

CHEM ABS, APS, MEDLINE, BIOSIS
SEARCH TERMS: ANTIBOD?, DSDNA, SANDWICH ASSAY,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF IMMUNOLOGICAL METHODS, VOLUME 132, ISSUED 1990, EMLEN ET AL., "A NEW ELISA FOR DETECTION OF DOUBLE-STRANDED DNA ANTIBODIES", PAGES 91-101, ESPECIALLY PAGES 91-93.	1-2
Y	JOURNAL OF LABORATORY CLINICAL MEDICINE, VOL. 97, NO. 6, ISSUED JUNE 1981, POPE ET AL., "IgG RHEUMATOID FACTOR: ANALYSIS OF VARIOUS SPECIES OF IgG FOR DETECTION BY RADIOIMMUNOASSAY", PAGES 842-853, ESPECIALLY PAGES 843-844.	1-2
Y	US, A, 4,391,904 (LITMAN ET AL.) 05 JULY 1983, SEE ENTIRE DOCUMENT.	1-2

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
03 AUGUST 1993	13 AUG 1993

Name and mailing address of the ISA/US	Authorized officer
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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF METHODS IN CELL AND MOLECULAR BIOLOGY, VOL. 2, NO. 3, ISSUED JUNE 1990, DENISE POLLARD-KNIGHT, "CURRENT METHODS IN NONRADIOACTIVE NUCLEIC ACID LABELING AND DETECTION", PAGES 113-132, SEE ENTIRE DOCUMENT	3-7