

312

Code
3405.00

SYNBIOTICS CORPORATION
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SAN DIEGO, CA 92127

OUTLINE OF PRODUCTION
CANINE LYMPHOMA MONOCLONAL ANTIBODY, Murine Origin
Product Code - 3405.00

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ESTABLISHMENT LICENSE NUMBER 312

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CANINE LYMPHOMA MONOCLONAL ANTIBODY, Murine Origin
OUTLINE OF PRODUCTION

I. Ascites Animals

- A. Description: Balb/C or Balb/C X Swiss F1 or immunodeficient mice; approximately 15 grams, in good health.
- B. Treatment Prior to Injection: Mice are admitted, quarantined, and cared for in conformance with 9CFR 117. Approximately two to six weeks prior to injection with hybridoma cells, each mouse is given pristane intraperitoneally.
- C. Care after Injection: Mice are held in approved cages in mouse rooms in conformance with 9CFR 117 and 108.

II. Antigen: Canine Lymphoma Cell Line 17-71.

A. Antigen Composition and Character

- 1. Cell Line: A canine lymphoma cell line established from a dog confirmed by histopathology to have multicentric lymphoma.
- 2. Source/Accession Date: [REDACTED] (b)(6)
[REDACTED] (b)(4)
- 3. Strain: The canine lymphoma cell line as originally developed was identified as 17-71.
- 4. Proportions: 100% Canine Lymphoma Cell Line 17-71.

B. Identification Methods

- 1. Confirmed to have canine lymphoma characteristics by histopathology.

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C. Virulence

1. Virulence: The cell line is not virulent.

D. Purity

1. Established by histopathology as a lymphoma of canine origin.

E. Passages

1. Master Cell Stock: 2 passages from receipt at Synbiotics.
2. Production Cell Stock: Not to exceed 10 passages from MCS.

F. Attenuation

1. N/A

G. Growth Containers

1. MCS is expanded in cell culture .

H. Media

1. Dulbecco's minimum essential medium (DMEM) plus fetal bovine serum (FBS).

I. Preparation

1. Cells are stored frozen in liquid nitrogen until used for testing of lymphoma antibody.

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III. Immunization of Animals: Preparation of Monoclonal Antibody

A. History of Hybridoma Used for Production

1. The hybridoma cell line used for production was developed [REDACTED] (b)(4)
[REDACTED] (b)(4)
[REDACTED] (b)(4)
2. The hybridoma/monoclonal antibody designated 231 was selected for the final product (MAb 231).
3. The hybridoma cell line was received by Synbiotics from [REDACTED] (b)(6) [REDACTED] (b)(4)
[REDACTED] (b)(4)

B. Hybridoma 231 was expanded in cell culture flasks to establish the master cell stock.

1. Master cell stock is designated [REDACTED] (b)(4) and was frozen within 2 passages after receipt at Synbiotics.
2. Cell reconstituted from the master or working cell stocks for injection into mice are within 20 passages of the master cell stock.
3. Antibody from the hybridoma cell line is tested for identity using [REDACTED] (b)(4)
4. Cells derived from the master cell stock have been tested for sterility and freedom from mycoplasma and extraneous viral contamination.

C. Ascites Production

1. An appropriate number of hybridoma cells are removed from the liquid nitrogen freezer and expanded in cell culture flasks that contain DMEM plus FBS.

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2. The required number of hybridoma cells are transferred to a centrifuge tube and pelleted by centrifugation.
3. Hybridoma cells are resuspended in an appropriate volume of Dulbecco's PBS for injection into mice.
4. Approximately two to six weeks after injection of pristane, each mouse is injected intraperitoneally with hybridoma cells.
5. Approximately two to three weeks after injection of hybridoma cells, ascitic fluids are collected.
6. After collection, tubes containing ascites fluids are centrifuged in a refrigerated centrifuge. Upon completion of centrifugation, all visible solids are removed. Ascites fluids are decanted into labeled containers and frozen at -20°C.
7. After thawing ascites fluids are (b)(4) and filtered. (b)(4) is either purified from ascites immediately or ascites is stored frozen.

IV. Preparation of Product

A. Antibody Purification

(b)(4)

(b)(4)

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(b)(4)

(b)(4)

B. Preservatives: N/A

C.

(b)(4)

D. Disposition of Unsatisfactory Material

1. All unsatisfactory material is discarded in accordance with VS Memo 800.56 and all applicable federal, state, and local regulations pertaining to the disposal of waste and maintenance of environmental quality.

E. Serial Size

1. Average Volume: 5 Liters
Maximum Volume: 10 Liters

V. Testing

A. Purity

1. Final container samples of each serial are tested for sterility as prescribed in 9CFR 113.26

B. Safety

1. Final container samples of each serial are tested for safety as prescribed in 9CFR 113.33(b)

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2. [REDACTED] (b)(4)
- [REDACTED] (b)(4)
- [REDACTED] (b)(4)

C. Potency

1. Protein Content: Bulk or final container samples of each serial are tested for protein content by ultraviolet spectroscopy using absorbance at 280 nm. [REDACTED] (b)(4) [REDACTED] (b)(4) The reference standard and each serial will be tested in quintuplet.
2. ELISA Potency Assay: Specific activity of bulk or final container samples of each serial is confirmed by demonstrating binding to [REDACTED] (b)(4) [REDACTED] (b)(4) The binding ratio of duplicate samples from each serial is compared to a reference standard. If the relative potency of the serial when compared to the standard reference does not equal or exceed 0.95, the serial is unsatisfactory. However, if the initial test shows a relative potency of less than 0.95, the serial may be retested two additional times. If the arithmetic mean of the three tests does not equal or exceed 0.95, the serial is unsatisfactory.

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Protocol for (b)(4) ELISA Potency Test

a. Materials:

(b)(4) Conjugate Stock
(b)(4) - Reference Antibody, Serial #EE002
(b)(4) On test antibody
10 mM Borate Buffer, pH 9.6
Blocking solution: 1%BSA, 10% sucrose in PBS pH
7.4
1771 lymphoma target cells
CL/MAb 231 ELISA diluent reagent
Chromogen reagent
Substrate reagent
Immulon II Removawell Test strips (12 wells per
strip)
Well holder

b. Reagent Formulation

1.) (b)(4)
The diluent used in the (b)(4) ELISA
assay is prepared as follows:

Sodium Phosphate, Monobasic	0.26 grams
Sodium Phosphate, Dibasic	1.15 grams
Sodium Chloride	8.75 grams
Phenol	1.00 grams
Ferrous Sulfate	0.0277 grams
Gentamycin Sulfate	1.0 ml
Deionized water	QS to 1000 ml

Adjust the pH of the solution to 7.2 ± 0.2 .
Filter through a 0.2 micron filter. Add SAS
cut normal mouse IgG (dialyze against PBS
prior to use to remove Ammonium sulfate) to a
concentration of 0.1mg IgG/ml of diluent.
Diluent is usually prepared in batches of 250
ml.

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- 2.) Chromogen reagent
Formulation of the Chromogen is as follows:
To 775 mls of deionized water add 13.6 grams of Sodium Acetate Trihydrate and 2.8 grams of Citric Acid Monohydrate. Mix thoroughly until salts are dissolved. pH of solution should be 4.9 - 5.1. Filter through 0.2 micron filter. Measure 750 mls of this solution into a clean container. To this container, slowly add 225 mls of Dimethyl formamide (DMF).

To 25 mls of DMF add 0.215 grams of 3,3',5,5' Tetramethylbenzidine (TMB). Stir until TMB is dissolved. Add the solution of TMB-DMF to the container of buffer/DMF. Mix thoroughly. Determine the OD 285 of the solution by making a 1:40 dilution (1.95 mls of water + 0.05 ml of TMB). The OD should be 0.49-0.54. Store at 2 - 7°C.

- 3.) Substrate reagent
Formulation of the Substrate is as follows:
- | | |
|---------------------------|---------------|
| Potassium Phosphate, | |
| Dibasic, anhydrous | 5.7 grams |
| Citric Acid Monohydrate | 3.6 grams |
| Benzoic Acid, Sodium Salt | 1.0 grams |
| Deionized water | QS to 1000 ml |

pH of the solution should be 4.9-5.1. Add 0.55 grams of Urea Peroxide and mix until dissolved. Filter through a 0.2 micron filter. Store at 2 - 7° C.

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4.) (b)(4) - HRP Conjugate Stock Preparation

a.) Materials Needed:

(b)(4) antibody
Boehringer Mannheim HRP
Sodium Bicarbonate (NaHCO_3)
Sodium Carbonate (Na_2CO_3)
Sodium Meta-Periodate (NaIO)
Sodium Borohydride (NaBH_4)
Sephadex G-25, Fine
Saturated Ammonium Sulfate
Glycerol

b.) Reagent Preparation:

Prepare 0.1M sodium bicarbonate by adding 0.84 g NaHCO_3 to 100 mls distilled water. Prepare 0.01M sodium carbonate by adding 1.18 g NaHCO_3 and 0.64 g Na_2CO_3 to 2 liters of distilled water. Adjust pH to

(b)(4)

c.) Periodate Activation of HRP:

Weigh out 5 mg HRP. Dissolve the HRP in 0.5 ml of 0.1M NaHCO_3 in a glass vial covered with foil. Prepare a 0.02M sodium meta periodate solution by dissolving 15 mg NaIO in 3.5 ml distilled water. While stirring the HRP solution, add 0.5 ml of the sodium periodate solution dropwise. Mix for 5 minutes and incubate at room temperature without stirring for 2 hours.

d.) Preparation of Antibody: (This step can be done during the above incubation period).

(b)(4)

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(b)(4) SAS-cut antibody against the 2 liters of 0.01M sodium carbonate, pH 9.6 for 2 hours at room temperature. Remove the antibody from dialysis and measure the volume. Calculate the amount of G-25 needed by adding the total volume of the HRP solution to the total volume of the dialyzed antibody solution. Multiply the resulting number by 0.16 to determine the number of grams of G-25 necessary.

- e.) Combination of Reactants:
Add the calculated amount of G-25 to the HRP solution. Immediately add the antibody. Mix on rocker mixer overnight at 2-7° C.
- f.) Reduction of Conjugate:
Filter G-25 from conjugate using a syringe and a 5 micron Acrodisk. Rinse sephadex with a small amount of PBS until HRP is flushed through. Measure volume. Sodium borohydride will be added to the conjugate solution in two steps. Calculate amount of 5 mg/ml NaBH to add at each step.

Conj. vol. _____ x 0.05 =
_____ ml NaBH (First addition)

Conj. vol. _____ x 0.15 =
_____ ml NaBH (Second addition)

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Prepare NaBH solutions immediately before use by adding 5 mg NaBH to 1 ml distilled water. Mix to dissolve. Add the volume of NaBH calculated for the first addition to the conjugate solution while mixing. Incubate for 30 minutes. Add the calculated volume of NaBH (freshly prepared) to the conjugate solution while mixing. Incubate for 60 minutes.

- g.) Ammonium Sulfate (SAS) precipitation of Conjugate:
Measure the volume of the conjugate. Add an equal volume SAS to the conjugate solution dropwise while mixing. Mix for 2 hours (conjugate can be stored at 2 - 7 °C overnight). Centrifuge conjugate for 15 minutes at 10,000 rpm. Decant the supernatant and discard. Resuspend the pellet in PBS, pH 7.4 at 1/2 the volume of the conjugate prior to the sodium borohydride reduction. Dialyze conjugate against PBS for 2 hours at room temperature or overnight at 4°C. Remove conjugate from dialysis and add an equal volume of glycerol. Filter conjugate-glycerol mixture through a 0.2 um Acrodisk into a clean glass vial and store at -20°C.
- 5.) Preparation of Coated Wells:
Thaw aliquot of Reference Antibody, Serial #EE002. Obtain 1 vial of new lot

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of (b)(4) on test. Prepare a 1:1000 dilution of the reference antibody by adding 10 μ l of antibody to 10 mls of 10 mM borate buffer. Prepare a 1:1000 dilution of the on-test antibody in the same manner. Coat the wells of eight removawell strips by adding 100 μ l of reference antibody to each well. Repeat the process with the on-test antibody. Incubate the wells containing the antibody solution overnight at 2 - 7°C. After incubation with the antibody solution, empty the wells and fill each well with 200 μ l of blocking solution (1% BSA, 10% sucrose in PBS pH 7.4). Incubate for 6 - 8 hours at room temperature. Remove the blocking solution from the wells and dry the wells for 18-24 hours at room temperature in a laminar flow hood. Dried wells should be sealed with parafilm or plastic wrap and stored at 2 - 7° C for up to six months.

6.) Preparation of Antigen:

a.) (b)(4)

Prepare the following antigen dilution buffer:

0.24 g Tris Base
0.88 g Sodium Chloride
3 mLs of NP₄₀
QS to 100 mls in deionized water.

Adjust pH to (b)(4)

Dilute the (b)(4) by adding 1 part of the cell pellet to 49 parts of the dilution buffer. Vortex 15 seconds and mix for at least 15

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minutes on rocker until centrifuged. Centrifuge 10 minutes at 10,000 rpm. Pool supernatants and mix 10 minutes. Aliquot in 0.5-1.0 ml volumes. Store at -20°C or -70°C .

- b.) Working solution: Thaw antigen aliquot. Vortex 15 seconds. Dilute 1:10 by adding $200\mu\text{l}$ of antigen to 1.8 mls of ELISA diluent reagent. Use same day and store at 4°C until used. Mix for 5-10 minutes on rocker mixer immediately prior to pipetting into wells. Discard unused portion.

- 7.) Preparation of Conjugate
Dilute the (b)(4) Conjugate Stock 1:6000 by adding $5\mu\text{l}$ of stock to 30 mls of ELISA diluent reagent. Mix on a rocker for at least 15 minutes. Diluted conjugate can be stored for up to 7 days at $2 - 7^{\circ}\text{C}$.

c. Assay procedure:

- 1.) Set up a plate with twelve on-test antibody coated wells and twelve reference antibody coated wells using the following configuration:

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	1	2	3	4	5	6
A						
B	T	T	T	R	R	R
C	T	T	T	R	R	R
D	T	T	T	R	R	R
E	T	T	T	R	R	R
F						
G						

- 2.) Add 100 ul (b)(4) working solution to rows B and C. Add 100 μ l ELISA diluent reagent to rows D and E. Cover plate with plate sealer and incubate 30 minutes at 37⁰C. Empty wells and blot once on paper towel; do not wash wells. Add 100 ul diluted (b)(4) (b)(4) conjugate to all wells. Cover plate with plate sealer and incubate 30 minutes at 37⁰C. Empty wells and blot. Wash 10 times with deionized water by filling each well with 250 μ l (2X with multichannel pipet set at 125 ul) beginning with row E and proceeding through row B. Empty wells and repeat 9 times blotting plate after final wash. Add 100 ul premixed TMB and UP (equal volumes) to each well using a multichannel pipet beginning with row B and proceeding through row E. Incubate 15 minutes at room temperature. Read plate at a test wavelength of 630nm and a reference wavelength of 490 nm using position A-1 as a blank.
- 3). Calculations:
For each well/sample combination calculate the mean optical density and the %CV of the six wells:

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- 1) Reference Antibody:Antigen
(wells B4,B5,B6,C4,C5,C6)
- 2) Reference Antibody:Diluent
(wells D4,D5,D6,E4,E5,E6)
- 3) On-Test Antibody:Antigen
(wells B1,B2,B3,C1,C2,C3)
- 4) On-Test Antibody:Diluent
(wells D1,D2,D3,E1,E2,E3)

For a valid test, the average OD of the Reference Antibody/Antigen wells must be at least (b)(4) of the Reference Antibody/Diluent wells. The %CV for all four groups of wells should not exceed 10%. If any of these criteria are not met, the test is a No Test and should be repeated.

Using the protein content determined in V.C.1., calculate the relative protein of the on-test antibody to the reference antibody as follows:

- A)
$$\text{Rel. Protein} = \frac{\text{Protein conc. of On-Test Ab}}{\text{Protein conc. of Reference Ab}}$$

Perform the following additional calculations:

- B) For the reference antibody, subtract the mean OD of the diluent wells from the mean OD of the antigen wells.

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- C) For the on-test antibody, subtract the mean OD of the diluent wells from the mean OD of the antigen wells.

To determine the RELATIVE POTENCY,

(b)(4)

(b)(4)

d. Acceptance Criteria

1. Final product will contain not less than (b)(4)
2. Final product will demonstrate a (b)(4)

Any serial which fails to satisfy these requirements may be retested twice. The average of these three (3) tests will be used to determine final acceptability.

VI. Post Preparatory Steps

- A. Form and Size of Final Containers: Six ml glass vials with rubber stopper and aluminum seal.
- B. Filling: Vials are filled using sterile techniques in a vertical flow HEPA filtered hood. Fill volumes are 4.7 to 4.9 ml per vial.
- C. Samples: Samples for submission to NVSL and for holding in reserve are collected and stored in

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accordance with 9CFR 113.3. Two vials are submitted to NVSL for concurrent testing. A minimum of six vials are held as retention samples. At least two of these vials will be retained throughout dating plus six months and will be available upon request for further APHIS testing.

- D. Expiration Date: The expiration date for the product will be 36 months from the date of initiation of the last satisfactory potency assay. The expiration date is subject to confirmation in accordance with 9CFR 114.13.
- E. Recommendation: (b)(4) is recommended as adjunct therapy in the management of canine lymphoma. The recommended dose is (b)(4) divided equally and administered over five consecutive days via slow intravenous infusion.
- F. Confidentiality: The following parts, sections, and paragraphs are considered confidential. Disclosure may cause harm to the competitive position of the submitter:

I, II, III, IV and V

Because of the foregoing, the firm feels disclosure of the above citations should be only to duly authorized officials of APHIS and then only in connection with their administration of the Virus-Serum-Toxin Act and such official business as may be pertinent.

Mary Anne Williams
Alternate APHIS Liaison

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SUMMARY OF CHANGES

Pages 7 - 17, V.C.2.

Revised to describe ELISA potency assay and protocol.

Page 18, VI.D.

Changed expiration dating from 24 to 36 months from the initiation of the last satisfactory potency test. ✓

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