

EFFECTIVE DATE	TITLE	METHOD NUMBER
INITIAL: 07.06.92	DETECTION OF β -LACTOGLOBULIN (BLG) AND SERUM ALBUMIN BY IMMUNODIFFUSION	NQA-08.2110
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KEY WORDS: Immunodiffusion, Ouchterlony, Hypoallergenic milks (LHA)		

I. SCOPE AND FIELD OF APPLICATION:

Description of a method for immunochemical determination of BLG and BSA in hypoallergenic milks.

II. PRINCIPLE:

Diffusion in an agar gel of BLG or BSA antigens of the product to the antisera (anti-BLG or anti-BSA). Formation of precipitate bands at the spots of the gel where the antigen and the antiserum meet at optimal concentrations. Staining of the latter to improve visibility. In case of positive reaction, semi-quantitative determination of the protein concentrations.

III. DEFINITION:

Immunodiffusion is defined as:

Any of several techniques for obtaining a precipitate between an antibody and its specific antigen by suspending one in a gel and letting the other migrate through it from a well, or by letting both antibody and antigen migrate through the gel from separate wells to form an area of precipitation.

IV. PRECISION:

Limit of detection: Presence of β -lactoglobulin (BLG) or serum lactalbumine (BSA) in a solution of 25 mg powder/ml of saline solution.

V. REFERENCES:

Primary: NESTEC, PLI-08.081. 1987-11-27 "Detection of β -lactoglobulin (BLG) and Serum Albumin (BSA) in Hypoallergenic Milks (LHA) by Immunodiffusion According to Ouchterlony"

VI. SAFETY PRECAUTIONS: (Refer to MSD Sheets before using chemicals)

Established laboratory safety requirements must be observed at all times, including the use of eye protection in laboratory work area.

- A. Acetic acid is a severe corrosive, which will cause extensive burns and damage to the eyes and skin. Upon contact, immediately flush with copious amounts of water. DO NOT breathe vapor. Use in a fume hood and wear Personal Protective Equipment (PPE) when handling.
- B. Ethanol is an extremely flammable liquid. Eliminate all possible sources of ignition. It is considered a poison and cannot be made non-poisonous. Wear Personal Protective Equipment (PPE) when handling.

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- C. Sodium Azide is a poison by ingestion, skin contact, intravenous and other routes. Has been shown to have profound hypotension and central nervous system effects. It is unstable, explosive, and reactive with several chemicals. Use Personal Protective Equipment (PPE) when handling. Fume hood should be used when dispensing.
- D. Additional chemicals associated with this method are not considered hazardous, however, Personal Protective Equipment (PPE) should be used when handling.
- E. Adequate precautions must be used when preparing agar by boiling. Use suitable glassware, and take extra precautions, since sodium azide is an ingredient in the preparation of the agar gel.

VII. SUPPLIES:

A. APPARATUS:

LKB:

1. Glass plates, No. 2117-402
2. Gel Bond Film for agarose gels 84 x 94 mm, No. 1850-102
3. Support for plates, No. 94924812
4. Template for double diffusion, No. 94924902
5. Levelling table 2117-404

BIO-RAD:

6. Gel Puncher, 3.0 mm, No. 170-4028
7. Magnetic stirrer, heated
8. Test tubes, SVL screw cap, Pyrex, length: 160 mm, diameter 16 mm, SVL 15
9. Paper for chromatography
10. Box, plastic, with lid, provided with a humid cloth, for storage of the gels
11. Vessel for washing the gels (e.g., staining box for electrophoresis)
12. Vessel for staining the gels (e.g., TLC chamber for nanoplates)
13. Crystallizing dish with spout, 140 x 75 mm, for destaining the gels
14. Volumetric flasks, class A, 20 and 1000 ml
15. Pipettes, Pasteur
16. Micropipettes, adjustable, 5 - 50 μ l and 50 - 200 μ l
17. Beakers, 2000 ml
18. Erlenmeyer flasks, 100 ml
19. pH-meter/mV-meter with a scale covering +700 mV
20. (Boiling) water bath
21. Water-jet pump

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B. CHEMICALS: (Refer to MSD Sheets)

1. Agar Noble, Difco No. 0142-01, Difco Laboratories, Detroit, USA
2. Di-Sodium hydrogen phosphate, ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), GR
3. Potassium dihydrogen phosphate, (KH_2PO_4), GR
4. Sodium chloride, GR
5. Sodium azide (NaN_3), extra pure
6. Ethanol (ethyl alcohol), about 95%, extra pure
7. Acetic acid glacial GR
8. Serva Blau R, extra pure (Coomassie Brilliant Blue R-250), Serva No. 35 051
9. β -Lactoglobulin bovine, cryst. extra pure, Serva No. 27 440
10. Serum albumin bovine (BSA) freeze-dried., p.a., Serva No. 11 924
11. Anti- β -Lactoglobulin freeze-dried, to be ordered from T-AQ; dissolve according to the manufacturer's instructions
12. Anti-BSA freeze-dried, to be ordered from T-AQ; dissolve according to the manufacturer's instructions
13. Deionized water (DW)

VIII. REAGENTS:
A. Phosphate - Sodium chloride buffer (PBS buffer), pH 7.2:

Into a 1000 ml volumetric flask, weigh 1.86 g sodium hydrogen phosphate, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.43 g potassium dihydrogen phosphate, KH_2PO_4 , and 8.8 g sodium chloride. Dissolve in DW and dilute to volume. The pH of the solution must be 7.2 (check with a pH-meter).

B. Agar gel:

Warning: Sodium azide (NaN_3) is toxic. Avoid contact with skin.

Into an adequate Erlenmeyer flask, weigh 1.5 g agar per 100 ml buffer. Under constant stirring (magnetic stirrer), add PBS buffer (VIII.A.) and bring slowly to the boil, so that the agar dissolves completely. Allow to cool slightly and add 0.02 g NaN_3 per 100 ml as bacteriostatic agent. As soon as azide has dissolved, dispense 14 - 15 ml portions of the agar solution into test tubes with cap. Allow to cool, stopper and store in the refrigerator.

C. Saline solution (washing solution):

Prepare an 0.9 % aqueous table salt solution.

If the gels remain more than 30 hours (weekend) in this solution, it is recommended to add 0.02 g NaN_3 per 100 ml solution.

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D. Staining solution:

In a 2000 ml beaker, dissolve, while stirring, 5 g Coomassie Brilliant Blue R 250 in 450 ml 96% ethanol, 450 ml DW, and 100 ml glacial acetic acid.

E. Destaining solution:

In a 2000 ml beaker, mix 450 ml 96% ethanol, 450 ml DW, and 100 ml glacial acetic acid.

F. Antisera:
1. Anti-BLG:

Dissolve the prescribed amount of freeze-dried antiserum in the prescribed amount of DW. This solution keeps for at least 3 weeks at 4°C.

2. Anti-BSA:

Prepare as outlined under VIII.F.1.

IX. PROCEDURE:
A. PREPARATION OF THE AGAR PLATES:

1. On a levelling table, place a defatted and dried glass plate. Cover it with a Gel Bond film, hydrophilic side upwards. Before use, check by means of a water drop that the hydrophobic side is actually underneath.
2. In a boiling water bath, completely liquefy the gel prepared under VIII.B. Quickly and carefully pipette 12 ml liquefied agar onto the middle of the plate, so that it covers the whole surface evenly. Allow to gelate for 5 minutes. Subsequently, place the plate in a closed box with humid atmosphere (wet cloth on the bottom of the box) and allow to solidify for 2 h at room temperature or for 1 h in the refrigerator. If required, the gels may be stored for 2 - 3 days in the humid chamber before use.
3. By means of the 3 mm puncher and the template, punch a central well surrounded by 6 analogous wells, at 5 mm from one another (use the part of the template with the greatest distance between wells).
4. Each gel support may contain a maximum of four such rosettes (see drawing, Appendix I).
5. By means of a Pasteur pipette connected to a water-jet pump, suck away all gel plugs.
6. Identify each gel by cutting out one or several edges with a knife (see drawing, Appendix II).

B. PREPARATION OF THE SOLUTIONS:
1. Product:

- a. Into a 100 ml Erlenmeyer flask, weigh 1.00 g powder and dissolve in 20.0 ml solution VIII.C. (about 50 mg/ml). For liquid hypoallergenic milks, dilute 7.87 g "Ready To Feed" in 12.13 ml solution VIII.C or 4.10 g "Concentrate" in 15.9 ml solution VIII.C.
- b. Subsequently, prepare a series of dilutions in such a way that each one contains half the amount of product of the previous dilution.

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- c. Into a test tube, introduce 1 ml of the solution prepared above. Pipette 0.5 ml into a second test tube and add 0.5 ml solution VIII.C. Mix. Pipette 0.5 ml of the first dilution into a third test tube and add 0.5 ml solution VIII.C. Prepare five dilutions in this way.
 2. Standard solutions:
Into two 50 ml volumetric flasks weigh 12.5 mg β -lactoglobulin and 12.5 mg serum albumin, respectively. Dissolve in solution VIII.C. and dilute to volume. Prepare five dilutions of each solution, as outlined under IX.B.1.
- C. IMMUNODIFFUSION:
1. For each product, perform an immunodiffusion test for BSA and BLG.
 2. For each series of analyses, perform an immunodiffusion test with both standard solutions.
 3. Into the central wells of the rosettes prepared under IX.A., introduce, by means of a micropipette, 10 μ l antiserum (VIII.F.1. or VIII.F.2.). Into the surrounding wells, introduce 10 μ l of the test portion or standard solution and of the five dilutions in the decreasing order of concentrations. Note the order chosen on the plans of the rosettes, see Appendix II.
 4. Place the plates into the closed box, at room temperature with humid atmosphere, and allow the antigens to diffuse towards the antisera (for at least 20 hours, until the precipitate bands of the standard solutions become visible).
 5. Wash the plates in a saline bath (e.g., staining box), preferably under magnetic stirring during 30 h. Replace the solution several times. Then wash for 2 - 3 h in DW.
 6. Remove the gels from the water and cover them with moistened Whatman No. 1 paper. Allow to dry overnight.
- D. STAINING AND DESTAINING:
1. Immerse the dried plates for about 10 minutes in the staining solution (VIII.D.). A chamber for small thin-layer chromatography plates is most practical. Remove the plates by means of tweezers.
 2. For destaining, place the plates in a bath (crystallizing dish) containing solution VIII.E. Replace this solution 2 - 3 times.
 3. Then rinse the gels with DW and wipe them with absorbing paper.
- E. EXPRESSION AND INTERPRETATION OF THE RESULTS:
1. Standard solutions:
The stained precipitates must be well visible (BLG at least up to the fourth; BSA, to the third dilution).

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2. Product:

The test is negative, if no precipitate band is visible.

The test is positive, if a precipitate band is clearly visible for one or several dilutions. See drawing, Appendix I.

Express the results as follows:

a. BSA

BSA negative in 50 mg/ml or in 500 mg/ml for liquid products.

or:

BSA positive in 50 mg/ml or in 500 mg/ml (i.e., a precipitate band is visible for the undiluted solution).

BSA positive in 25 mg/ml or in 250 mg/ml (i.e., a precipitate band is visible for the undiluted solution and for the first dilution), etc.

b. BLG

See IX.E.2.a.

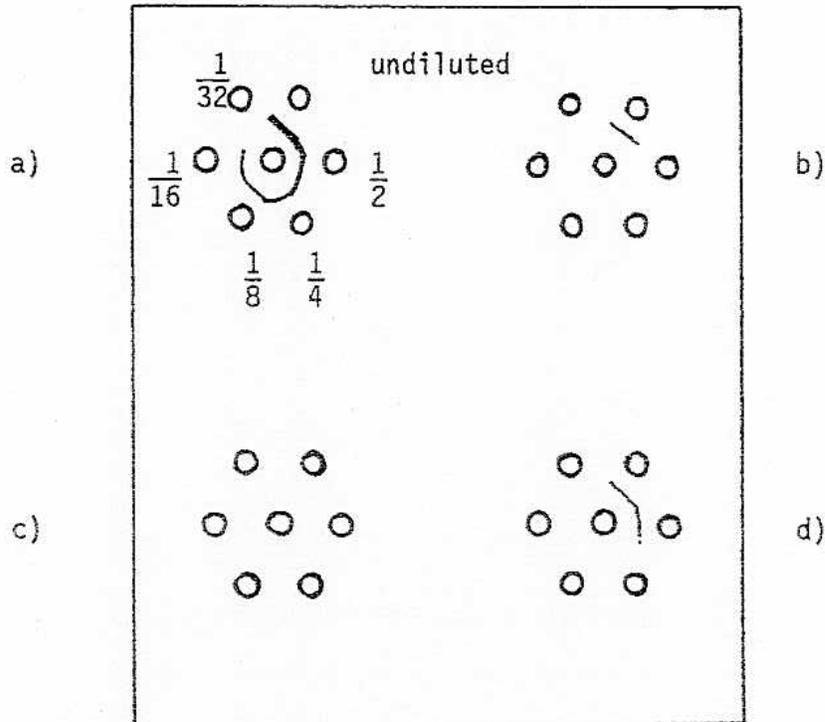
X. **INTERNAL CONTROL PLAN:**

Each laboratory must set up its own I.C.P., using the following guidelines. Frequencies can be adapted according to number of samples. Record results of I.C.P. in your Quality Assurance manual.

- A. All determinations must be carried out in duplicate.
- B. Parallel determination by two persons on the same product: once a month.
- C. Verification of balance(s) accuracy with appropriate weights: daily.
- D. Verification of balance(s) accuracy, maintenance contract: every 6 months.
- E. Calibration of pH-meter(s) with buffers (pH:4.00 & 7.00), (logsheet): daily.
- F. Verification of indicated shelf life of prepared solutions: once a week.

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APPENDIX I.

EXAMPLE OF GEL WITH STANDARD AND PRODUCT


- a) Standard
- b) Product: positive in 50 mg/ml
- c) Product: negative in 50 mg/ml
- d) Product: positive in 25 mg/ml

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APPENDIX II.
DIAGRAM OF THE ROSETTES
