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Immunoblotting: An Emerging Technique in Immunohematology

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ABSTRACT

Immunoblotting is a technique that use antibodies (or another specific ligand) to identify target proteins among a number of unrelated protein species. They involve the identification of protein targets via antigen-antibody-specific reactions. These techniques are generally comprised of four distinct phases: Electrophoretic separation of protein or of nucleic acid fragments in the sample, Transfer to and immobilization on paper support, Binding of analytical probe (antibodies or DNA) to the target molecule on paper, Visualization of bound probe. Immunoblotting methods include ELISA, Western blotting, Northern blotting, Southern blotting. ELISA is an immunological technique to detect the presence of an antigen, antibody, other proteins or glycoproteins in a given biological sample. Western blotting is also known as immune blotting or protein blotting which is based on the principles of immunochromatography where proteins were separated into polyacrylamide gel according to the isoelectric point and molecular weight. In the past, immunoblotting was used simply to detect a specific target protein in a complex mixture. Interpretation of blot data has been done in terms of fold changes in protein expression between samples. The calculations are based on the differential densitometry of the associated chemiluminescent and/or fluorescent signals from the blots and this now requires a fundamental shift in the experimental methodology, acquisition, and interpretation of the data. Here we summarize the complete Immunoblot workflow with a focus on sample preparation and data analysis for quantitative assessment.

INTRODUCTION:

Immunoblotting is a technique that use antibodies (or another specific ligand) to identify target proteins among a number of unrelated protein species. They involve the identification of protein targets via antigen-antibody-specific reactions. Immunoblotting techniques are generally comprised of four distinct phases: Electrophoretic separation of protein or of nucleic acid fragments in the sample, Transfer to and immobilization on paper support, Binding of analytical probe (antibodies or DNA) to the target molecule on paper, Visualization of bound probe. Immunoblotting methods include ELISA, Western blotting, Northern blotting, and Southern blotting[1].

ENZYME-LINKED IMMUNOSORBENT ASSAY:

ELISA is an immunological technique to detect the presence of an antigen, antibody, other proteins or glycoproteins in a given biological sample. The basis of this test is the ability of the antibody to bind specifically to a given antigen. This involves an antigen (or antibody) coated onto the substrate. The sample containing the antibody (or antigen) is added, which gets immobilized due to its interaction with the coated antigen (or antibody). Finally, the antibodies (primary or secondary antibodies) are added, which are conjugated to a detection system. Detection system consists of an enzyme capable of converting a substrate to a colored product. The colour change can be used to quantify the amount of antibody (or antigen) present in the sample[2].

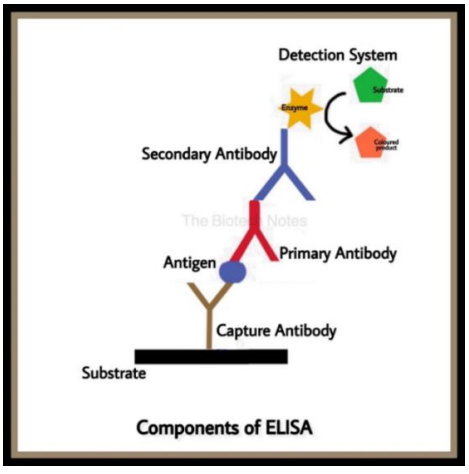

Figure:1 Components of ELISA

COMPONENTS OF ELISA:

1. Antigen:

Antigens present in the diagnostic samples are immobilized or coated directly onto the substrate or held by the capture antibody. In the cases where the **antibodies** have to be detected from the sample (serum, etc), the antigens are absorbed/ immobilised onto the substrate.

2. Capture Antibody:

The capture antibody is specific for the sample antigen. It binds and captures the antigen to be detected. The function of the capture antibody is to immobilise the antigen from the sample onto the substrate and prevent its wash-off.

3. Primary Antibody:

The primary antibody is used to detect the presence of the antigen. The primary antibody directly binds the antigen at an epitope different than the capture antibody. This antibody can be conjugated to an enzyme or a protein (biotin), which helps in detection.

4. Secondary Antibody:

A secondary antibody specifically binds to an untagged **primary antibody.** It is conjugated with the **detection system.** A single secondary antibody can be used for a range of primary antibodies, hence surpassing the need to conjugate each primary antibody with the detection system.

5. Detection system:

The detection antibody (either primary or secondary antibody) is linked to a detection system. This either involves an **enzyme** (linked directly or to streptavidin) and a substrate. The most commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP). β-galactosidase, acetylcholinesterase and catalase are also used[3].

TYPES OF ELISA:

1. Direct ELISA:

This is a one-step and most basic format. In this format, the **antigen** from the diagnostic sample is immobilized onto the substrate and detected with an antibody **(primary antibody)** conjugated to an enzyme (detection system).

2. Indirect ELISA:

This is a two-step method. In this, the **antigen** is immobilized to the surface. But the **primary antibody** is not conjugated with the detection molecule, but rather a **secondary antibody** specific for the primary antibody. The detection molecule is conjugated with the secondary antibody.

3. Sandwich ELISA:

This has a **capture antibody** coated onto the substrate. Then the **antigen** sample is added. If the antigen under investigation is present, the antigen gets immobilized. Then the primary antibody against another epitope of antigen is added, which is linked to the **detection system** and brings about change in the color of the substrate. The format may use a secondary antibody as well.

4. Competitive ELISA:

In this format, the **sample antigen** competes with a **reference antigen** for binding the same paratope on the antibody (conjugated with the detection system) present. The sample is incubated with the linked antibodies**,** this mixture is then introduced into the tube with reference antigens coated onto the substrate[3].

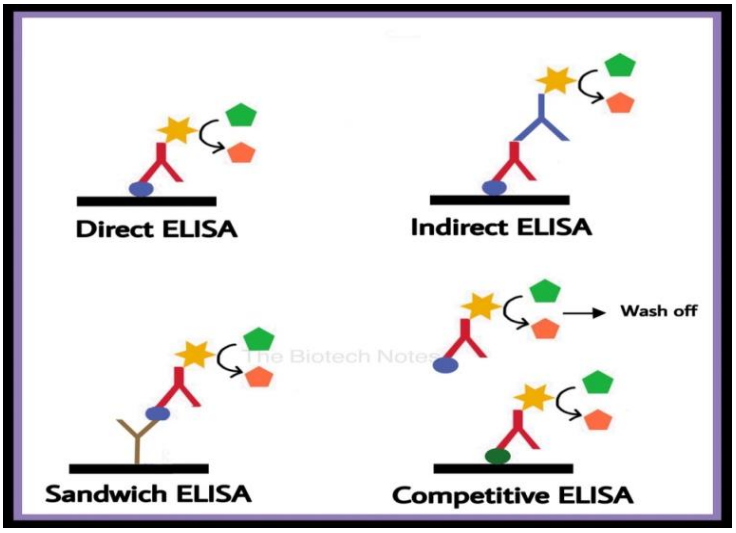

Figure:2 Types of ELISA

PROCEDURE:

ELISA is one of the easiest blood tests that can be carried out. It is rapid, quick and requires a blood sample of the patient. The entire procedure of ELISA is mentioned below.

- An antibody is attached to a polystyrene plate which is a solid surface and is attracted or has an affinity towards bacteria, other antibodies and hormones.
- A microtiter coated with antigen is filled with this antigen-antibody mixture after which free antibodies are removed by washing.
- A secondary antibody specific to the primary antibody is added which is usually conjugated with an enzyme.
- Free enzyme-linked secondary antibodies are removed by washing the plate.
- Finally, the substrate is added. The substrate is converted by the enzyme to form a colored product, which can be measured by spectrophotometry.

HCG protein which indicates pregnancy is detected by ELISA. A combination of blood or urine sample and purified HCG linked to an enzyme is added to the system. If HCG is absent in the test sample, then only the linked enzyme binds to the solid surface.

The more the substance of interest is present, the more reaction takes place and less of the linked enzyme binds to the solid surface. These reactions are indicated usually by a change in the colour of the solution[4].

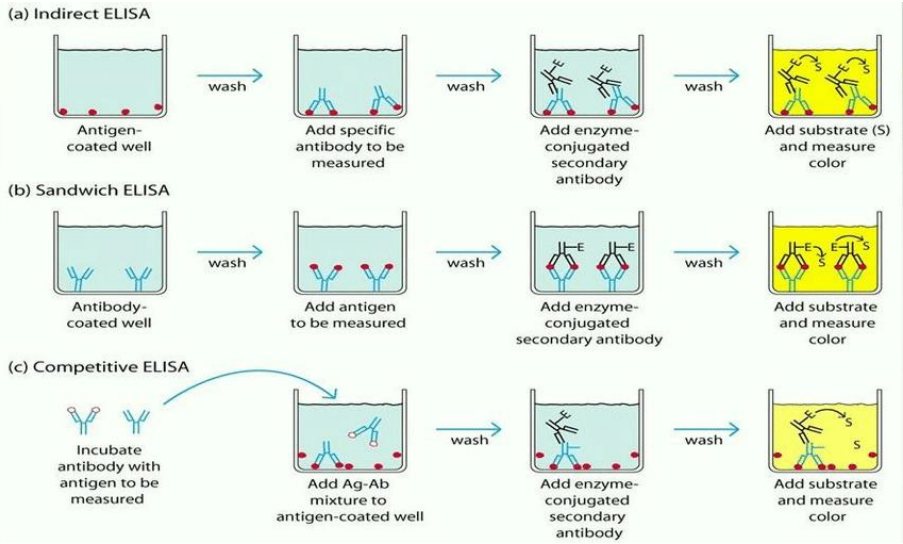

Figure:3 Steps involved in ELISA

DISEASES THAT CAN BE DIAGNOSED USING ELISA:

ELISA can be used to detect some of these conditions: Ebola, Pernicious anemia, AIDS, Rotavirus, Lyme disease, Syphilis, Toxoplasmosis, Zika virus, Carcinoma of the epithelial cells[4].

ADVANTAGES OF ELISA:

✓ Results fetched from ELISA give an accurate diagnosis of a particular disease since two antibodies are used.

✓ Can be carried out for complex samples as the antigen is not required to get purified to detect.

✓ It is highly responsive since direct and indirect analysis methods can be carried out.

✓ It is a rapid test, yields result quickly.

✓ Possible detection for ELISA ranges from the quantitative, semi-quantitative, standard curve, qualitative, calibration curve models etc.

✓ Easier to perform and uncomplicated process as compared to other assays which require the presence of radioactive materials [5].

APPLICATIONS OF ELISA:

ᔓ The presence of antibodies and antigens in a sample can be determined.

ᔓ It is used in the food industry to detect any food allergens present.

ᔓ To determine the concentration of serum antibodies in a virus test.

ᔓ During a disease outbreak, to evaluate the spread of the disease, e.g. during a recent COVID-19 outbreak, rapid testing kits are being used to determine the presence of antibodies in the blood sample[5].

WESTERN BLOTTING:

It is also known as immune blotting or protein blotting. It is used to detect the presence of a specific protein in complex mixtures by labeled antibodies. It is based on the principles of immunochromatography where proteins were separated into polyacrylamide gel according to the isoelectric point and molecular weight[6].

PROCEDURE:

Steps involved in Western blotting:

i.) Tissue preparation:

Samples may be taken from whole tissue, from cell culture, bacteria, viruses or environmental samples. In most cases, samples are solid tissues. First broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication and also by filtration and centrifugation. To encourage lysis of cells and to solubilize proteins, may be employed: detergents, salts, and buffers. To prevent the digestion of the sample by its own enzymes-antiprotease and phosphatase. To avoid protein denaturing-Tissue preparation is often done at cold temperatures.

ii.) Gel Electrophoresis:

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point, molecular weight, electric charge, or a combination of these factors. Commercially SDS-PAGE gel electrophoresis is used for proteins. Polyacrylamide gel: Polymerized gel: 1. Resolving gels made in 6%, 10%, 12%, 18%. 2. Stacking Gel up to 5%. The percentage chosen depends on the size of the protein. SDS-PAGE is a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility, to separate proteins according to their size, and no other physical features. SDS (the detergent soap) breaks up hydrophobic areas and coats proteins with negative charges thus overwhelming positive charges in the protein. Therefore, if a cell is incubated with SDS, the membranes will be dissolved, all the proteins will be solubilized by the detergent and all the proteins will be covered with many negative charges. They are traveling to the positive since they have a negative charge.If the proteins are denatured and put into an electric field (only), they will all move towards the positive pole at the same rate, with no separation by size. However, if the proteins are put into an environment that will allow different-sized proteins to move at different rates. The environment is polyacrylamide. The entire process is called polyacrylamide gel electrophoresis (PAGE). Small molecules move through the polyacrylamide faster than big molecules, big molecules stay near the well.

iii.) Transfer:

In order to make the proteins accessible to antibody detection they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). The membrane is placed on top of the gel, and a stack of filter papers is placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it.

iv.) Blotting:

Blotting is used to transfer the samples from the gel onto a membrane such as a nylon membrane or nitrocellulose membrane.

v.) Blocking:

Steps must be taken to prevent interactions between the membrane and the antibody (since the antibody is a protein itself). Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of Bovin Serum Albumin (BSA) or non-fat dry milk, with a minute percentage of detergent such as Tween 20.

vi.) Detection:

The membrane is "probed" for the protein of interest with a modified antibody that is linked to an enzyme (alkaline phosphatase or horse radish peroxidase), which when exposed to an appropriate substrate drives a colorimetric reaction and produces a colour.

vii.) Analysis:

Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis.

- Colorimetric detection
- Radioactive detection
- Fluorescent detection[6,-9]

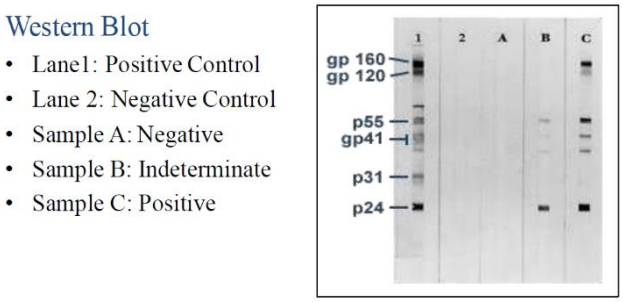

Figure:4 Western Blotting

APPLICATIONS OF WESTERN BLOTTING:

- The confirmatory HIV test employs a western blot to detect anti-HIV antibodies in a human serum sample.
- A western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').
- Some forms of Lyme disease testing employ western blotting.
- Western blot can also be used as a confirmatory test for Hepatitis B infection.
- In veterinary medicine, western blot is sometimes used to confirm FIV+status in cats [6-9].

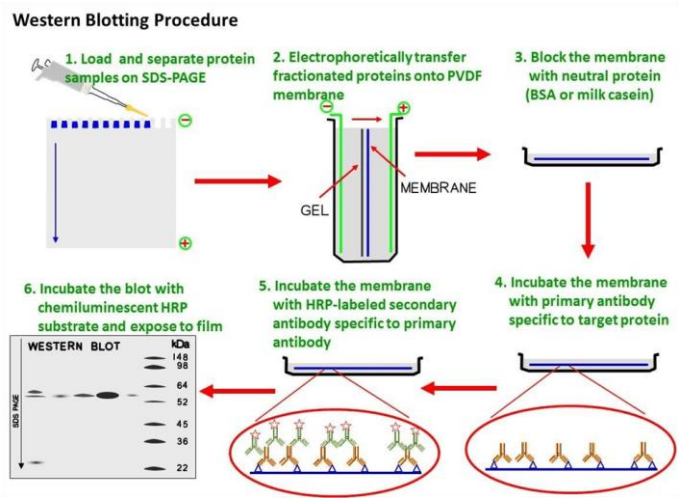

Figure:5 Steps involved in Western blotting

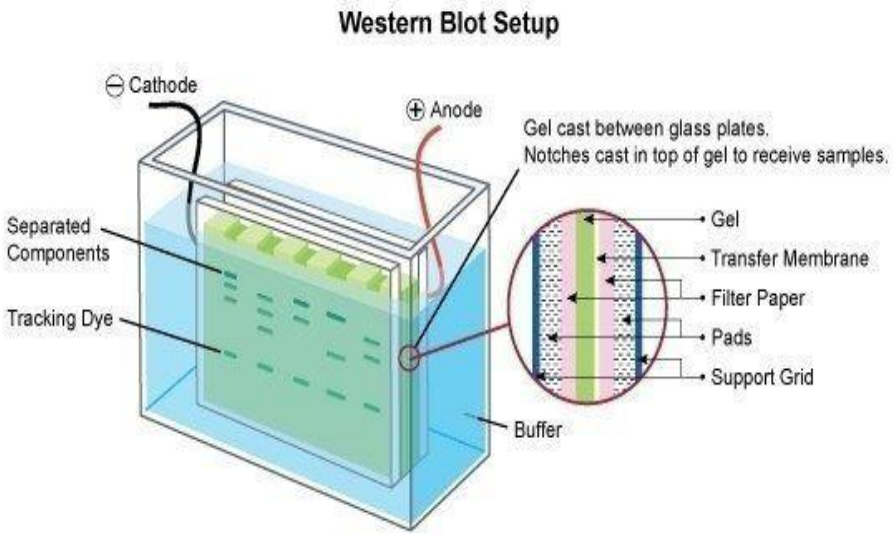

Figure:6 Western blot setup

SOUTHERN BLOTTING: (Restriction Fragment Length Polymorphism)

Used in molecular biology for the detection of a specific DNA sequence in DNA samples. Named when British Biologist Edwin Southern published it in 1975. Southern blotting founded on hybridization principle. Restriction endonuclease, which is an enzyme, is used to break the DNA into small fragments. These fragments are then separated using electrophoresis. The fragments achieved are then classified according to their size (kDa). Thus, DNA fragments are transferred to the blotting paper where it is incubated with highly selective probes. Probes can be selectively bound with a resolution of 1 in a million and the characteristics to bind to the intended target fragments[10].

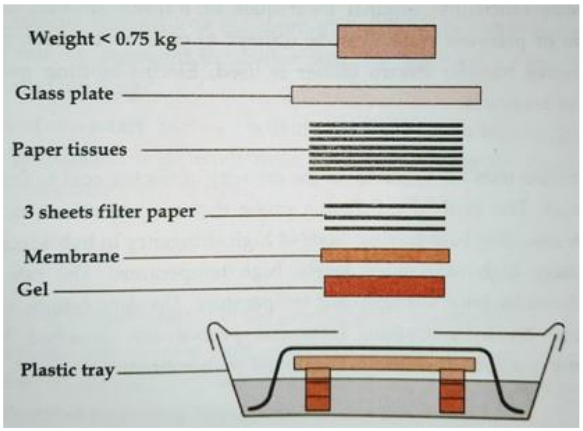

Figure:7 Typical Southern blotting assembly

PROCEDURE:

a) Extraction and purification of DNA from cells: DNA is first separated from target cells following standard methods of genomic DNA extraction and then purified.

b) Restriction Digestion or DNA Fragmentation: Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments. One or more restriction enzymes can be used to achieve such fragments.

c) Separation by Electrophoresis: The separation may be done by agarose gel electrophoresis in which the negatively charged DNA fragments move towards the positively charged anode, the distance moved depending upon its size.

d) Depurination: Partial depurination is done by the use of dilute HCl which promotes higher efficiency transfer of DNA fragments by it breaking down into smaller pieces.

e) Denaturation: DNA is then denatured with a mild alkali such as an alkaline solution of NaOH. This causes the double-stranded DNA to become single-stranded, making them suitable for hybridization. DNA is then neutralized with NaCl to prevent re-hybridization before addition of the probe.

f) Blotting: The denatured fragments are then transferred onto a nylon or nitrocellulose filter membrane which is done by placing the gel on top of a buffer saturated filter paper, then laying the nitrocellulose filter membrane on the top of gel. Finally, some dry filter papers are placed on top of the membrane. Fragments are pulled towards the nitrocellulose filter membrane by capillary action and result in the contact print of the gel.

g) Baking: The nitrocellulose membrane is removed from the blotting stack, and the membrane with single stranded DNA bands attached to it is baked in a vacuum or regular oven at 80 °C for 2-3 hours or exposed to ultraviolet radiation to permanently attach the transferred DNA onto the membrane.

h) Hybridization: The membrane is then exposed to a hybridization probe which is a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labeled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye.

i) Washing of unbound probes: After hybridization, the membrane is thoroughly washed with a buffer to remove the probe that is bound non-specifically or any unbound probes present.

j) Autoradiograph: The hybridized regions are detected auto radio graphically by placing the nitrocellulose membrane in contact with a photographic film that shows the hybridized DNA molecules. The pattern of hybridization is visualized on X-ray film by autoradiography in case of a radioactive or fluorescent probe is used or by the development of color on the membrane if a chromogenic detection method is used[11-15].

APPLICATIONS OF SOUTHERN BLOT:

- ✓ Identifying specific DNA in a DNA sample
- ✓ Preparation of RFLP (Restriction Fragment Length Polymorphism) maps
- ✓ Detection of mutations, deletions or gene rearrangements in DNA
- ✓ For criminal identification and DNA fingerprinting (VNTR)
- ✓ Detection and identification of trans gene in transgenic individual
- ✓ Mapping of restriction sites.
- ✓ Prognosis of cancer and prenatal diagnosis of genetic diseases.
- ✓ Determination of the molecular weight of a restriction fragment and measuring relative amounts indifferent samples[15].

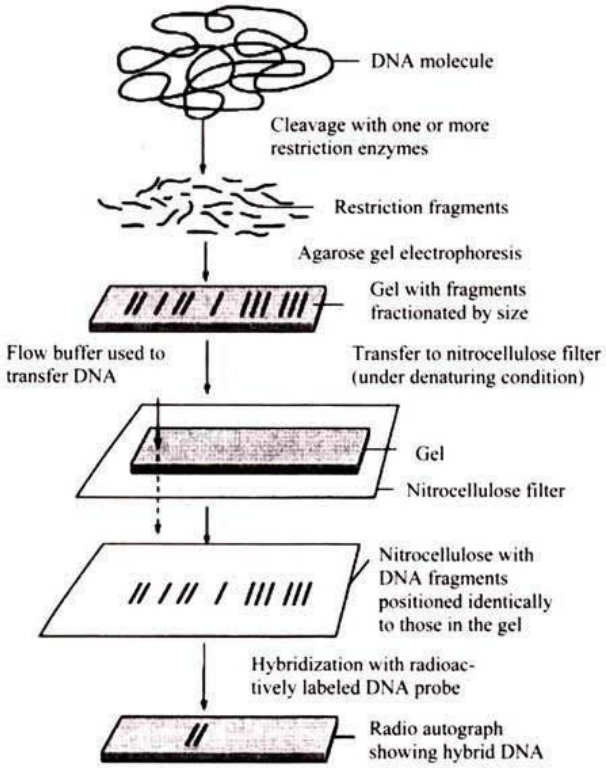

Figure: 8 Steps involved in southern blotting

CONCLUSION:

Immunoblotting is a technique that has been in practice for more than three decades that began as a means of detecting a protein target in a complex sample. Although there have been significant advances in both imaging and reagent technologies to improve sensitivity, dynamic range of detection, and the applicability of multiplexed target detection, the basic technique has remained essentially unchanged. In the past, immunoblotting was used simply to detect a specific target protein in a complex mixture. Interpretation of blot data have been done in terms of fold changes in protein expression between samples. The calculations are based on the differential densitometry of the associated chemiluminescent and/or fluorescent signals from the blots and this now requires a fundamental shift in the experimental methodology, acquisition, and interpretation of the data. Here we summarize the complete Immunoblot workflow with a focus on sample preparation and data analysis for quantitative assessment.

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