ARISE

African Research And Innovative Initiative For Sickle Cell Education

Session 7

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 824021

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Principles of IEF for newborn screening /protocols

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TRAIN- THE TRAINER

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TOPIC:NEWBORN SCREENING USING ISOELECTRIC FOCUSING (IEF) TECHNIQUE

- DEFINITION: Isoelectric focusing is the electrophoretic method that separates proteins (heamoglobins) according to the isoelectric points. The Isoelectric point is the specific PH at which the net change on the protein is zero.

  IEF is used for early infant diagnosis(EID) for babies with HIV positive mothers through DBS analysis.

  A protein with a positive net charge will migrate towards the cathode becoming progressively test positively charged as it travels through the PH gradient until it reaches a point that corresponds with its PH value.

- PRINCIPLE:-

  The principle of this essay is to separate haemoglobin by electrophoresis in a PH gradient set up between a cathode and anode. The haemoglobin samples are loaded unto a precast agarose gel(PH6.8.) when an electrical current is applied to the gel, the haemoglobin variants possessing individual isoelectric points migrate through the gel forming a stable PH gradient. When an individual variant's PH equals the corresponding PH in the gel, its migration stops as the charges on the haemoglobin variant is zero. The trichloroacetic acid(TCA) disrupt the tertiary structure of haemoglobin. Once disrupted, internal hydrophobic amino acids are exposed causing the haemoglobin to coagulate and fall out of solution.

The haemoglobin bands are visualized on the gel using a protein stain and their positions measured relative to the control bands

•PURPOSE: The purpose of this essay is to separate whole blood samples for the detection of normal and variant haemoglobin by isoelectric focusing (IEF) . This essay serves as an acid in the diagnosis of neonatal and adult haemoglobinopathies and is one of the three recommended techniques to confirm HbS,HbC,HbD,and HbE variants. Children born with sickle cell disease have an increased risk of infection, painful crises, and death. Diagnosis of sickle cell disease in a newborn baby leads to a better tyeatment of the newborn baby and good quality of life.

• STANDARD OPERATING PROCEDURE(SOP)

The procedures are as follows:-

1. Wear protective hand gloves
2. Put on laboratory coat.
3. Bring reagents to room temperature 30 mins prior to use because they are stored at 2-8^{0}c
4. Prepare samples from bloodspots on DBS (Guthrie) cards.
5. Allocate a well number for each specimen in the IEF worksheet.
6. Using manual puncher, punch 3mm holes from the DBS cards and place in the corresponding well of the microtitre plate.
7. Add 80 microlitre of Hb Elution solution to each well.
8. Cover the microtitre plate and allow to stand for 30 mins at room temperature and mix

To ensure even consistency using the sonic bath

9. Start electrophoretic machine ensuring water is at the required level by turning on the power switch at the back and the On switch at the front.
10. Pipette water unto the centre of the cooling plate using syringe and position the gel in the center of the cooling plate between lanes 4 and 14
11. Remove the topmost sheet on the gel and blot gently with the blotting paper the same size as the gel.
12. Place 3wicks on another plate; one for cathode and two for anode right on their positions. Anode on both sides and cathode at the center.
13. Place rough surface of wicks up.
14. Wet wicks with their various solutions
15. Blot wicks separately with blotting paper, anode alone, cathode alone.
16. Place wicks on gel rough surface down according to their positions i.e. cathode at the middle and anode on the both sides.
17. Place two templates at each side of the cathode wick and blot gently to remove air bubbles using automatic pipette.
18. Load control and samples according to the worksheet(approx 5.0 microlitre for control and neonatal samples,& approx3.0 microlitre for adult samples).controls are FASE (FASE is applied at the beginning and the end), FADC(FADC is applied at the middle).
19. Put on both cathode and anode rods in place i.e. anode(back position)while cathode(front position)

20. Place the cover over electrophoretic tank.

21.press`run" for 1 ½ hrs at voltage 1500v.

22. Press stop and off light from the tank.

23. Open and remove wicks ensure to clear both anode and cathode wicks separately with paper towel soaked in deionised water and dry.

24. Remove gel from tank.

25. Place gel on gel shaker and wash with deionised water for 5mins

26.Decant and cover the gel with deionised water for 10 mins

27. Decant and place the gel in a staining plate or rack.

28. Cover the gel with 250ml of 10% TCA for 10 mins i.e. (fixing).

29.Decant and cover gel with deionised water and wash for 1hour.

Staining preparation:-

Pipette the following:-

i. Stain buffer- 15ml

ii. Gel stain concentrate- 27ml into 500ml measuring cylinder.

iii. Make it up to 150ml with deionised water.

iv. Add 3ml of stain activator

30.Pour off the water on the gel.

31. Cover gel with prepared stain for 2 ½ mins
32. Discard supernatant
33. Cover gel with deionised water to stop reaction
34. Discard supernatant
35. Wash with deionised water for 10 mins
36. Discard supernatant
37. Wash with deionised water for 1 ½ hrs
38. Discard supernatant
39. Place gel in dryer for 1 ½ hrs
40. Read controls first.

- CALCULATION OF RESULTS

1. Measue the gel distance in mm,from the A to S, A to C, A to D and A to E bands(use bands of the control samples: FACE and FADC)
2. Calculate the correction factor to correct for gel to gel variations by dividing the reference distance by the measured gel distance to get the variants relative position.
3. Reference distance is measured in mm from HbA. Example of reference distance.

A to F 3.0
A to S 8.5
A to C 16.0

For ~~example~~

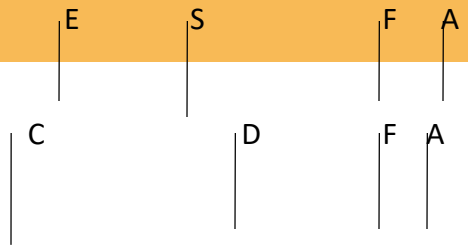
a. if the gel distance from A to S is 8 mm, the calculation will be as follows. 8.5mm/8.0 mm = 1.06

b. If the gel distance measured from A to C is to 18.5, the calculation will be as follows 16mm/18.5= 0.86

Note: for any variant measured below 10 mm, apply the AS correction factor.

For any variant greater than (>) 10 mm,apply the AC correction factor.

INTERPRETATION OF BANDS:-



RECORDING METHOD:-

Results are recorded as follows,

a. In IEF worksheet.
b. Laboratory register book
c. CIHP register book
d. In the computer system using BLISS (Basic Laboratory Information System).

CIHP register book:- this include the

- Serial Number
- Child's name
- Unique ID number (i.e. numbers that are given to patients from different wards, they could be out patient or in patient) e.g.
- OPE- Out Patient Expose

- TROUBLESHOOTING

1. Inadequate blotting may result in the haemoglobin variant bands running into each other.
2. Excess or insufficient anode and cathode solution added to the wick may cause the haemoglobins to travel incorrectly.
3. Burning along the edges of the gel may be due to too much water beneath the gel.
4. wavy or uneven band may be due to too much sample supplied at the wick.

- QUALITY ASSURANCE:-

1. Internal quality assurance
2. External quality assurance

1. Internal quality assurance:- FASE and FADC controls are used as internal quality assurance. FASE is used twice i.e one at the begining and one at the end of run. FADC is used at the middle of the run. FASE can also be used at the middle if space permits.
2. External quality assurance: this involves preparation of IEF worksheet which is as follows:-

- All patient samples awaiting IEF results are logged on a worksheet.
- The details of the neonatal specimen, i.e the name, laboratory number etc and controls are recorded.
- The lot number of the gel ,lot number of each reagent used and their expiry are recorded as well.

ADVANTAGE OF IEF:

IEF gives a better resolution and separate more variants compared to cellulose acetate.

High resolution depends on

i. The PH gradient
ii. The thickness of gel
iii. The time of electrophoresis
iv. The applied voltage
v. Diffusion of the protein into the gel

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