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INSTRUCTION MANUAL OF LDH KIT

(KT-LD001/ KT-LD005/ KT-LD010)

Manufactured and Marketed by:

TechResource

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Kit components

Sr. No.	Components	Store at
1.	30% Acrylamide soln.	4°C
2.	Separating buffer	4°C
3.	Stacking buffer	4°C
4.	TEMED	4°C
5.	APS (powder)	4°C
6.	Loading dye buffer	4°C
7.	Sealing agar	4°C
8.	5X tank buffer	4°C
9.	Substrate buffer	4°C
10.	NAD (oxidiser) 10 mg/mL	-20°C
11.	PMS (stabilizer) 1 mg/mL	-20°C
12.	NBT (electron acceptor) 1 mg/mL	-20°C
13.	D/W	4°C

Equipments and consumables required but not provided in the kit:

-Vertical Electrophoresis unit, (maximum size 7 x 8.5)
-Power supply
-Freshly minced chicken (brain/liver/ heart/ kidney)
-Tissue homogenizer
-Clean scissors & forceps
-Centrifuge (for microfuge tube)
-Glass pipettes (1, 5, 10 ml) 1 each
-One 25 ml measuring cylinder
-Two 250 ml beakers
-Micropipettes-
0.5-10 µL
5-50 µL
10-100 µL
100-1000 µL
-Tips
-Refrigerator
-37C incubator

Preparation instructions:

1X tank buffer:

1X tank buffer is to be prepared from 5X tank buffer by diluting with D/W.

Table 3:

	1 Expt.	5 Expts.	10 Expts.
D/W	120 mL	600 mL	1200 mL
5X Tank buffer	30 mL	150 mL	300 mL
Total	150 mL	750 mL	1500 mL

Store 1X tank buffer at 4°C.

10% APS:

In 1.5 mL tube, weigh 0.1 g of APS from a microfuge tube labelled as APS and add 1 mL D/W. Mix to dissolve and store in dark at 4°C. (*Prepare just before use*)

Table 1: For separating gel (10%)

Chemicals	For Slimpage (Gel size- 4X8.5 cm)	For Microkin/Ecopage (Gel size- 8.5X7.5)
D/W	2.4 mL	4.5 mL
Separating buffer	1.52 mL	2.78 mL
30% Acrylamide soln.	2.0 mL	3.7 mL
10% APS soln.	30 µL	60 µL
TEMED	4 µL	7 µL
Total	6 mL	11 mL

Note: Add TEMED and APS at the time of pouring the solution in the sandwich since polymerization starts immediately on addition of these chemicals

Table 2: For stacking Gel (4%)

Chemicals	For Slimpage (Gel size- 4X8.5 cm)	For Microkin/Ecopage (Gel size- 8.5X7.5)
D/W	1.2 mL	2.4 mL
Stacking buffer	0.5 mL	1 mL
30% Acrylamide soln.	0.27 mL	0.54 mL
10% APS soln.	10 µL	20 µL
TEMED	2 µL	5 µL
Total	2 mL	4 mL

Note: Add TEMED and APS at the time of pouring the solution in the sandwich since polymerization starts immediately on addition of these chemicals

Activity staining solution:

Mix 5 mL substrate buffer, 1 mL NADH oxidizer, 0.25 mL PMS stabilizer and 2.5 mL NBT. Mix and store in dark place at -20 °C.

Protocol summary:

1) Clean and dry the apparatus.
2) Assemble the unit and seal with agarose.
3) Prepare the sample
4) Place the comb and mark the level till which the separating gel needs to be poured.
5) Prepare separating gel as per table no.1
6) Pour the separating gel till the level marked in above step and overlay with D/W.
7) Allow to polymerize for 30-45 mins.
8) Prepare stacking gel as per table 2.
9) Flick off the overlay D/W.
10) Pour the stacking gel and immediately insert the comb.
11) Allow to polymerize for 10-15 mins.
12) Mark the wells and remove the comb gently.
13) Fill the apparatus with chilled 1X tank buffer.
14) Before loading the samples, add loading buffer to it (1:1).

Centrifuge the extract again for 5 min.

15) Load the samples. Connect to a power supply and carry out electrophoresis at required Voltage.
16) Switch the power supply off when the blue tracking dye reaches the bottom of the gel.
17) Prepare activity staining solution.
18) Remove the gel from the assembly and Stain the gel with staining solution.

20) Interpretation of zymogram.

LDH zymography

Introduction:

Lactate dehydrogenase (LDH) exists in five forms (i.e. Isozymes). Each one has a slightly different structure that makes their separation feasible. Zymography is a poly-acrylamide gel electrophoretic technique, for qualitative detection of enzyme activity. LDH isozymes are separated on a PAGE and activity staining is performed on the gel to visualize separated isozymes. In LDH activity staining, the enzymes present on in the gel react with substrate and a series of reactions occur to form a purple coloured precipitate. The reactions occur as follows:

Lactate + $NAD^+ \leftrightarrow$ Pyruate+ NADH + H^+

NADH + PMS (oxidized) $\leftrightarrow$ NAD^+ + PMS (Reduced)

PMS (Reduced) + NBT (oxidized) $\leftrightarrow$ PMS (oxidized) + NBT (Reduced) Purple precipitate

[PMS: PhenazineMethosulfate, NBT:Tetranitrobluetetrazolium]

Theory:

A solution of acrylamide and bisacrylamide is polymerized to from polyacrylamide gel. Acrylamide alone forms linear polymers. The bisacrylamide forms crosslinks between polyacrylamide chains. The gel pore size depends on the ratio of acrylamide to bisacrylamide, and the concentration of acrylamide. Higher the concentration of acrylamide smaller the pore size. Polymerization of acrylamide and bisacrylamide is induced by addition of ammonium persulphate (APS), which spontaneously decomposes to form free radicals. TEMED is a free radical stabilizer which improves the acrylamide polymerization. The standard SDS-PAGE system is a discontinuous gel with an upper stacking gel and lower resolving gel that have different polyacrylamide concentrations and pH values. The stacking gel has a lower percentage of polyacrylamide allowing proteins to move quickly and stack into a tight band before entering resolving gel which has higher percentage of polyacrylamide for separation. Glycine in the upper buffer will exist as both a zwitterion with net charge of 0 and a glycinate ion with a charge of -1. When glycinate anions enter the stacking gel of pH 6.8 along with proteins, chloride ions, bromophenol blue, they shift their equilibrium to the zwitterion form and become neutral. The loss of charge causes them to move very slowly in the electric field. On the other hand chloride ions which are highly negatively charged move quickly. Hence a moving boundary region is rapidly formed, where the chloride ions are in the front and the relatively slow glycinate ions are in the trailing region. All of the proteins in the gel sample have intermediate mobility, so when the two fronts sweep through the sample well, the proteins are concentrated into the narrow zone between the chloride ions and glycinate ions. There is a remarkable change in mobilities of glyciante ions and proteins when they enter the resolving gel of pH 8.9. Once in the resolving gel, glycinate ions become negatively charged and migrate much faster than proteins whereas migration of proteins is slowed down due to the smaller pore size (sieving effect) of the gel and are subsequently separated by their sizes, with the larger molecules moving slower.

Detailed Protocol:

Assembling and sealing the apparatus:

1. Rinse all items, beakers, measuring cylinders, pipettes, funnels and electrophoretic apparatus with distilled water; flick dry and use (As the chlorine in tap water will adversely affect the stacking). Prepare 1X tank buffer as mention in the instruction. Cover with a plastic sheet and chill in the refrigerator.
2. Assemble the PAGE apparatus (As described in the instrument manual).
3. If you are using gel casting system for Microkin then skip to next step (sealing with agar).
4. Melt the sealing agar in a hot water bath. Seal the bottom of the sandwich by pouring 5 mL of the molten agar into the trough in the lower tank, also seal the slot of the spacer with hot agar. Let the agar set for 10 mins.

Sample preparation:

1. Mince the freshly removed chicken brain, kidney, liver & heart in a tissue homogenizer using distilled water.(For every gram of chicken tissue use 2 mL of distilled water).Make a fine slurry.
2. Transfer 1 mL of the slurry to 1.5 mL microfuge tubes and keep it out at room temp. (RT) for 30 min to allow enzyme to diffuse out of the cells.
3. Centrifuge for 10 mins. at 2,000 rpm. Collect the supernatant in fresh 1.5 mL tubes and re-centrifuge as above. Collect the supernatant and store the extract at RT

Casting the Gel:

1. Place the comb in assembled apparatus. Using a permanent marker, mark the level till which the separating gel needs to be poured. (This can be about 1 cm below the level of the bottom of the wells of the comb). Remove the comb from the apparatus.
2. In a clean beaker add the components of the separating gel as per Table 1.

(TEMED should be kept in the fridge, add TEMED and APS at the time of pouring the solution in the sandwich since polymerization starts immediately on addition of these chemicals.)

3. Use a beaker or a pipette to pour the separating gel solution in the assembled cassette till the level marked in step 5.
4. Using a 1 mL pipette carefully overlay D/W over the separating gel. This is done to prevent oxidation of the separating gel buffer which may result in incomplete polymerization. Do not use butanol as it damages the apparatus.
5. Let the gel polymerize for 30-45 mins. Do not shift the apparatus.
6. Flick off the overlay water, blot out any remaining water with filter paper making sure no lint is left behind. The water remaining behind could dilute out the stacking gel that is being poured.
7. In a clean beaker add the components of the stacking gel as per table 2. Use a pipette to pour the stacking gel. and immediately insert the comb.
8. Make sure no air bubbles are trapped in between the wells. If there are any air bubbles remove the comb halfway and insert the comb again.
9. Mark the position of the wells with a permanent marker.
10. Let the gel polymerize for 10-15 mins.
11. At the end of the period for polymerization slowly pull out the comb ensuring that the wells don't collapse
12. Wash the wells with D/W to remove any unpolymerized acrylamide.
13. Fill the cathodic and anodic chamber with chilled 1X tank buffer.

Running the gel:

1. Before loading the samples, add loading buffer to it (1:1). Centrifuge the extract again for 5 min.
2. Load different volumes of sample (between 15-40 uL) per well. Ensure that no precipitate is transferred to the wells.
3. Connect the apparatus to power supply. Start the power supply. Adjust the voltage and current as per your requirement. (For a small apparatus of 8.5 x 7 cm adjust to a constant voltage of 100 V)
4. Switch the power supply off when the blue tracking dye reaches the bottom of the gel.
5. Remove the sandwich from the apparatus and carefully prise open the sandwich. Mark the orientation of the gel by cutting off a small triangular portion from the lower left-hand corner of the gel.
6. If you are using glass plates and not the fibre glass plate provided with the Microkin, then skip to step 7.
7. The polyacrylamide gel attaches itself firmly to the fibre-glass plate. To peel gel from this plate, insert the gel-detacher under the gel, and while doing so pour water along its edge. Slide the gel-detacher towards the opposite edge, and pour water behind the gel-detacher to ensure that the gel does not re-adhere to the plate.

Activity staining:

1. Prepare the working staining solution in a tray, by mixing substrate buffer, electron acceptor, stabilizer and oxidizer (as mentioned in the preparation).
2. Carefully slide the gel into the tray containing staining solution. Incubate for 10-15 mins. in the dark at 37C or until bands develop. Observe the zymogram for blue bands against white background.
3. Discard the stain and store the zymogram in 7.5% acetic acid.