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Starch Gel Electrophoresis of Enzymes—A Compilation of Recipes

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INTRODUCTION

The technique of starch gel electrophoresis of enzymes with specific staining for activity in the gel, the so-called zymogram method developed by Hunter and Markert (1957), has found many research applications. Screening studies, comparing relatively large numbers of enzymes among a variety of tissues and organisms, are finding increasing use in research on population genetics, taxonomy, etc. The methods presently available are scattered through the literature. This compilation is published in response to numerous requests from investigators in many fields.

Most of the methods outlined here have been used primarily for tissue extracts from mammalian species. A few have been developed only on plants or lower animals, but this is not to say that they will not work on higher organisms. The buffer systems have been for the most part empirically arrived at. All are probably subject to improvement.

The techniques of making the gels and carrying out the electrophoresis are not described here. These have been amply presented in a number of publications. The vertical system is essentially that described by Shaw and Koen (1968*b*). The horizontal system is essentially as described by Beckman and Johnson (1964). The horizontal method has the advantage of employing simpler and less expensive apparatus, and it can be run at room temperature using ice trays to keep the gels cold. For many enzyme systems, it provides as good results as the vertical.

Both Connaught starch (Connaught Laboratories, Toronto) and Electrostarch (Otto Hiller Company, Madison, Wisconsin) will give satisfactory results with either technique. Unfortunately, different batches of starch may produce varying results, both in rate of migration and in resolution; also, some batches contain substances

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inhibitory to certain enzyme activities. For consistent results, a single batch should be employed throughout a study.

In the interest of saving space, the chemical principles underlying the methods have not been described. Nor is the reference list intended to be exhaustive; it merely attempts to give primary credit for certain of the methods. Most of the methods are, of course, adaptations of standard histochemical techniques.

The individual staining mixture recipes are arranged by groups according to the general class of reaction (dehydrogenases, hydrolases, etc.), and alphabetically within each group. The appropriate gel and electrode buffers for each enzyme are indicated by number, and the buffer recipes are outlined in Table I. All the enzymes are listed in Table II, in the order in which their recipes occur. Stock solutions used in certain buffer systems and staining solutions are listed in Table III.

HYDROLASES, LYASES, AND TRANSFERASES

Acetyl- and Pseudocholine Esterases

Method of Gomori (cited by Pearse, 1961).

Buffer System II

Stain Buffer: 0.022 M glycine—0.075 maleic acid, *p*H 6.0	
Glycine	1.65 g
Maleic acid	8.71 g
1.0 M NaOH	15 ml
H_2O to	1 liter
Stain:	
A. $CuSO_4 \cdot 5\ H_2O$	0.15 g
$MgCl_2 \cdot 6\ H_2O$	0.51 g
Na_2SO_4 (anhydrous)	34.1 g
Glycine–maleic buffer, *p*H 6.0 (stain buffer)	100 ml
B. Acetylthiocholine iodide	200 mg
C. 40% Na_2SO_4 (saturated)	100 ml
D. 0.01 M $(NH_4)_2S$ (3 ml of 23.7% solution/100 ml stain buffer)	100 ml
E. Tetraisopropylpyrophosphoramide (0.5 mg/ml)	0.5 ml
F. 1.5 *bis*-(4-trimethyl ammonium phenyl) pentan-3-one diiodide (0.05 mg/ml)	1 ml

Add reagent B to solution A. Incubate gel in this at 37 C for 1 hr. Pour off and rinse gel in solution C. Let the gel stand in solution D for 10 min or until dark brown bands are visible. Pour off D and fix.

For acetyl choline esterase only: To the mixture of A and B add also E. Develop as before.

For pseudocholine esterase only: To the mixture of A and B add F. Develop as before.

No.	Buffer	Electrode				Gel			
		Components (per liter)				Components (per liter)			
		1	2	3	pH	1	2	3	pH
I	0.155 M tris–0.043 M citric acid (tris citrate)	Tris 16.35 g	Citic acid 9.04 g	—	7.0	Dilute 66.7 ml of electrode buffer to 1 liter			7.0
II	0.3 M borate	Boric acid 18.55 g	NaOH 2 g	—	8.0	Boric acid 1.86 g	NaOH 0.48 g	—	8.5
III	0.5 M tris–versene–borate	Tris 60.6 g	Boric acid 40.0 g	Na_2EDTA 6.0 g	8.0	Tris 6.06 g	Boric acid 6.00 g	$Na_2EDTA \cdot 2 H_2O$ 0.60 g	8.0
IV	0.341 M borate	Boric acid 21.1 g	NaOH 5.32 g	—	9.0	Boric acid 2.11 g	NaOH 0.6 g	—	9.0
V	0.687 M tris–0.157 M citric acid	Tris 83.2 g	Citric acid 33.0 g	—	8.0	Tris 2.77 g	Citric acid 1.10 g	—	8.0
VI	0.23 M arsenate	KH_2AsO_4 41.4 g	KOH 250 ml of 1 M KOH	—	8.0	KH_2AsO_4 0.69 g	KOH 4 ml of 1 M KOH	—	8.0
VII	0.2 M acetate	Sodium acetate 27.2 g	Acetic acid about 12 ml	—	4.6	Dilute 100 ml of electrode buffer to 1 liter			4.6
VIII	0.41 M citrate	Citric Acid 86.2 g	NaOH 600 ml of 2 M NaOH	—	7.0	Histidine 0.78 g	—	—	7.0
IX	0.5 M tris–HCl	Tris 60.0 g	HCl 150 ml of 1 M HCl	NaCl 5.8 g	9.0	Dilute 100 ml of electrode buffer to 1 liter			9.0
X[a]	0.03 M lithium hydroxide–0.19 M boric acid	$LiOH \cdot H_2O$ 1.2 g	H_3BO_3 11.89 g	—	8.1	Citric acid 1.44 g Tris 5.59 g	$LiOH \cdot H_2O$ 0.12 g	H_3PO_3 1.89 g	8.2
XI	0.0546 M tris–0.2454 M borate	Tris 6.61 g	Boric acid 15.17 g	—	7.5	Tris 0.12 g	Boric acid 1.79 g	—	7.5
XII	0.233 M tris–0.086 M citric acid	Tris 27.0 g	Citric acid 18.1 g	—	7.0	35 ml of electrode buffer diluted to 1000 ml			7.0
XIII	0.378 M tris–0.165 M citric acid	Tris 45.8 g	Citric acid 34.7 g	—	6.0	33.3 ml of electrode buffer diluted to 1000 ml			6.0
XIV	0.214 M K_2HPO_4–0.027 M citric acid	K_2HPO_4 29.1 g	Citric acid $\cdot H_2O$ 5.7 g	—	7.0	K_2HPO_4 1.06 g	Citric acid $\cdot H_2O$ 0.254 g	—	7.0
XV	0.163 M phosphate	K_2HPO_4 (anhyd.) 19.0 g	KH_2PO_4 (anhyd.) 7.3 g	—	7.0	K_2HPO_4 (anhyd.) 0.45 g	KH_2PO_4 (anhyd.) 0.39 g	—	7.0
XVI	0.5 M phosphate	K_2HPO_4 (anhyd.) 87.0 g	KH_2PO_4 (anhyd.) 68.0 g	—	7.0	Dilute 100 ml of electrode buffer to 1 liter			7.0
XVII	0.272 M phosphate	K_2HPO_4 (anhyd.) 31.5 g	KH_2PO_4 (anhyd.) 12.4 g	—	7.0	K_2HPO_4 (anhyd.) 0.751 g	KH_2PO_4 (anhyd.) 0.646 g	—	7.0
XVIII	0.1 M tris–0.1 maleic–0.01 M EDTA	Tris 12.1 g	Maleic acid 11.6 g	$Na_2EDTA \cdot 2 H_2O$ 3.72 g $MgCl_2 \cdot 6 H_2O$ 2.03 g	7.4	Dilute electrode buffer 1 : 10 times with H_2O			7.4
XIX	0.2 M phosphate	460 ml of 0.2M $NaH_2PO_4 \cdot H_2O$ (27.8 g/liter)	40 ml of 0.2 M $Na_2HPO_4 \cdot 7 H_2O$ (53.65 g/liter)	—	5.8	Dilute 50 ml of electrode buffer to 1 liter			5.8
XX	0.2 M phosphate	255 ml of 0.2 M $NaH_2PO_4 \cdot H_2O$	245 ml of 0.2 M $Na_2HPO_4 \cdot 7 H_2O$	—	6.8	Dilute 65 ml of electrode buffer to 1 liter			7.0

[a] To make gel for buffer system X, make the stock solution of 0.008 M monohydrate citric acid and 0.05 M tris and mix this buffer in 1 : 10 proportion.

Table II. List of Enzymes and the Buffer Systems Used for Them

Group	Enzymes	Buffer system	
		Vertical	Horizontal
Hydrolases, lyases, and transferases	Acetyl- and pseudocholine esterases	II	II
	Acid phosphotase	I, XI	I
	Aconitase	V	—
	Aldolase	III, V	III
	Alkaline phosphatase	I, II	I
	Carbonic anhydrases	IV, II	—
	Esterase	II	II
	Fructose 1,6-diphosphatase	V	—
	Beta-glucuronidase	VII	—
	Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)	XVII	—
	Leucine aminopeptidase (LAP)	II	II
	Pepsinogen	III, V	—
	Peptidase	I	—
	Ribonuclease	I, XIV	—
Oxidases and peroxidases	Catalase	II, V	—
	Ceruloplasmin	II	—
	Monoamine oxidase	V	—
	Peroxidase	I, V	I
	Tyrosinase	II	—
Dehydrogenases	Alcohol dehydrogenase (ADH)	I, XVI	I
	D (−)-Hydroxybutyrate dehydrogenase (HBDH)	XVIII	
	Galactose 6-phosphate dehydrogenase (gal-6 PDH)	I, III	—
	Glucose 6-phosphate dehydrogenase (G6PDH)	III	III
	Glutamate dehydrogenase (GDH)	I, III	—
	Glutathione reductase	VIII	—
	Glyceraldehyde 3-phosphate dehydrogenase (G 3 PDH)	I, XVII	—
	α-Glycerophosphate dehydrogenase (αGPDH)	I, III	I, III
	Hexose 6-phosphate dehydrogenase	III	—
	Isocitrate dehydrogenase (IDH)	I, XIV	I
	Lactate dehydrogenase (LDH)	I, VIII, XIV	I, X
	Malate dehydrogenase (MDH)	I	I
	6-Phosphogluconate dehydrogenase (6PGD)	III	III
	Retinol dehydrogenase (RDH)	VI	—
	Sorbitol dehydrogenase	XIV	—
	Xanthine dehydrogenase (XDH)	III	III
Miscellaneous	Adenylate kinase (AK)	I	—
	Aromatic amino acid transaminase	III	—
	Creatine kinase (CK)	I	—
	Fumarase	III	III
	Glucosephosphate isomerase (6PI)	XX	—
	Glutamate-oxaloacetate transaminase (GOT)	I	I, X
	Hexokinase (HK)	I	—

Table II (*continued*)

Group	Enzymes	Buffer system	
		Vertical	Horizontal
	Phosphoglucomutase	I	I
	Pyruvate kinase (PK)	IX	—
	Thymidine kinase	XIX	—
	Triosephosphate isomerase (TPI)	I	—
Bacterial enzymes	Alanine dehydrogenase	I, III	—
	L-leucine dehydrogenase	III	—
	Peroxidase and catalase	II	—
	Pyrophosphatase	II	—

Table III. Stock Solutions Used in Certain Buffer Systems and Staining Solutions

Solution	Molarity	*p*H	Buffer systems or enzymes
KOH	1 M		Buffer system VI
NaOH	2 M		Buffer system VIII
$Na_2HPO_4 \cdot 12\ H_2O$	0.2 M		Buffer system XIX
$Na_2HPO_4 \cdot 7\ H_2O$	0.2 M		Buffer system XX
HCl (conc.)	1 M		Buffer system IX
HCl	0.1 M		Pepsinogen
NaOH	1 M		Acetyl and pseudocholine esterases, esterases
Na_2SO_4 (saturated)	40%		Acetyl and pseudocholine esterases
Veronal buffer	0.1 M	9.0	Carbonic anhydrases
α,β-Naphthyl acetate	1%		Esterases
Tris–HCl	0.5 M	7.1	Aldolases, esterases, adenylate kinase, creatine kinase, xanthine dehydrogenase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, LDH, IDH, G3PDH, glucose 6PDH, ADH, galactose DH, MDH, HK
NaOH	0.2 M		Aminopeptidases
Phosphate	0.1 M	7.0	Pepsinogen, catalase, tyrosinase, RDH, fumarase, GOT
Acetate buffer	0.05 M	5.0	Acid phosphatase, peroxidase
Tris buffer	0.005 M	7.5	HGPRT
KI	0.09 M		Catalase
$Na_2S_2O_3 \cdot 5\ H_2O$	0.06 M		Catalase
Na acetate	0.1 M	5.7 4.7	Ceruloplasmin
$CaCl_2$	0.1 M		Peroxidase
H_2O_2	3%		Peroxidase
Catechol	0.01 M		Tyrosinase
L-proline	0.1 M		Tyrosinase
NaCN	0.1 M		Galactose dehydrogenase
$(NH_4)_2SO_4$	1 M		Gal-6PDH
Phosphate buffer	0.5 M	7.0	Glutamate dehydrogenase
Na_2CO_3	1 M		LDH
Na_2EDTA	0.1 M		Triophosphate isomerase

Acid Phosphatase

Modification of the method of Allen *et al.* (1963).

Buffer System I or XI

Stain Buffer: 0.05 M acetate, *p*H 5.0	
Na acetate · 3 H_2O	6.8 g
HCl (1 N)	14.8 ml
H_2O to	1 litre
Adjust *p*H with 0.1 N HCl.	
Stain:	
Na α-naphthyl acid phosphate	100 mg
Stain buffer	100 ml
Black K salt	100 mg

Incubate gel at 37 C until bands appear. Wash and fix.

Aconitase

Modified method of Koen (1969).

Buffer System V

Starch Gel: Add 30 g sucrose per 600 ml of gel. Add 10 mg $NADP^+$ just before deaeration step.

Stain:

B.	NBT	30 mg
	PMS	2 mg
	$NADP^+$	25 mg
	0.5 M phosphate buffer, *p*H 8.0	8 ml
	0.1 M *cis*-aconitate, *p*H 7.0 or 8.0	8 ml
	H_2O	24 ml
C.	Gelatin	500 mg
	H_2O	3 ml
	Solution B	2 ml
	Isocitric dehydrogenase	0.5 ml (12 1/2 units)

Mix solution C in a dish warmed to 37 C. Then place gel with cut surface down. Pour the rest of solution B and 40 ml H_2O on top and incubate.

Aldolase

Buffer System III or V

Stain:

Na_4 fructose 1,6-diphosphate · 5 H_2O	545 mg
Glyceraldehyde 3 PD	100 units (0.6 ml)

NAD^+	50 mg
NBT	30 mg
PMS	2 mg
0.5 M tris–HCl buffer, *p*H 7.1	10 ml
$Na_2AsO_4 \cdot 7H_2O$	150 mg
H_2O	90 ml

Incubate at 37 C until bands appear. Wash and fix.

Alkaline Phosphatase

Method of Boyer (1961).

Buffer System I or II

Stain:

β-Naphthyl Na phosphate	50 mg
Fast blue RR	50 mg
$MgSO_4 \cdot 7\ H_2O$	123 mg
H_2O	100 ml

Incubate at 37 C until blue bands appear. Wash and fix.

Beta-Glucuronidase

Method of Fondo and and Bartalos (1969).

Buffer System VII

Substrate Buffer:

6-bromo 2-naphthl beta-D-glucuronide	30 mg
Absolute ethanol	10 ml
Phosphate–citrate buffer *p*H 4.95	20 ml
H_2O	70 ml

Stain:

Fast Blue B	100 mg
0.02 M phosphate buffer (*p*H 7.5)	100 ml

Dissolve fast blue and filter the stain solution.

Incubate gel in substrate buffer at 37 C for 4 to 6 hr (or overnight). Rinse in tap water and immerse in freshly prepared stain solution until blue bands appear. Wash twice in cold distilled water and rinse in 0.1% acid solution.

Carbonic Anhydrases

Method of Pihar (cited by Tashian, 1969).

Buffer System II or IV

Stain:

1% bromthymol blue (in 0.1 M veronal buffer, *p*H 9.0)	20 ml
CO_2	

Cover gel surface for 15 min (or until gel becomes blue) with filter paper soaked in bromthymol blue. Remove paper and direct CO_2 through rubber tube onto the surface of gel. Yellow zones of carbonic anhydrase activity appear against the blue background.

To slow down the enzyme activity, the procedures should be carried out in a cold room.

Esterase

Buffer System II

Substrate: 1% α,β-naphthyl acetate

α-Naphthyl acetate	1 g
β-Naphthyl acetate	1 g
Acetone	50 ml
H_2O	50 ml

Stain:

Fast blue RR	100 mg
0.5 M tris–HCl, *p*H 7.1	10 ml
1% α,β-naphthyl acetate	3 ml
H_2O	87 ml

Incubate at room temperature until blue bands appear. Wash and fix.

Fructose 1,6-Diphosphatase

Method of Stout (1969).

Buffer System V

Stain:

0.5 M tris–HCl, *p*H 7.5	10 ml
TPN	15 mg
$MgSO_4 \cdot 7\ H_2O$	250 mg
PMS	2 mg
NBT	20 mg
2-Mercaptoethanol	0.002 ml
Fructose 1,6-phosphate	20 mg
H_2O to	100 ml

Add just before use: 40 units phosphohexose isomerase and 40 units glucose 6-phosphate dehydrogenase. Incubate gel at 37 C until dark bands appear. Wash and fix.

Hypoxanthine-Guanine Phosphoribosyl Transferase (HGPRT)

Method of Der Kaloustian *et al.* (1969).

Buffer System XVII

Stain:

Tris buffer (*p*H 7.4)	5 mM (2 ml)
$MgCl_2$	2 mM (2.33 mg/2 ml)
Guanine	0.1 mM (0.03 mg/2 ml)
PRPP (phosphoribosyl pyrophosphate)	0.55 mM (0.52 mg/2 ml)

Apply DEAE paper soaked with stain solution to the gel, making sure to have a close contact. Incubate gel and paper together for 90 min at 37 C in moist chamber. Peel off the paper from the gel and suspend in 2–3 liters of 8 mM tris solution (*p*H 9.5), with continuous stirring for 4 hr at room temperature. Dry the paper at room temperature and place on X-ray film. Press tightly between two pieces of cardboard sandwiched by two pieces of glass. Expose film for 4 days. Dark bands appear in developed film.

Leucine Aminopeptidase (LAP)

Modification of the method of Smith and Rutenberg (1966).

Buffer System II

Stain Buffer: Tris–maleate buffer, *p*H 6.0

A. Tris	24.2 g
Maleic acid	23.2 g
B. 0.2 M NaOH	26 ml

Mix:
50 ml of solution A
26 ml of solution B
H_2O to 200 ml

Stain:

Tris–maleate buffer, *p*H 6.0	50 ml
H_2O	50 ml
Black K salt	50 mg
L-leucyl β-naphthylamide	20 mg

Incubate gel at 37 C until dark bands appear. Wash and fix.

Pepsinogen

Modification of the method of Hanley *et al.* (1966).

Buffer System III or V

Stain Buffer: 0.1 M phosphate buffer, *p*H 7.0

KH_2PO	13.6 g
1.0 M NaOH	59 ml
H_2O to	1000 ml

Stain:

A. 0.1 M HCl	100 ml
B. Amidoschwarz dye	1 g
Bovine albumin	0.5 g
Stain buffer, *p*H 7.0	100 ml

Peptidase

Modification of the method of Lewis and Harris (1967).

Buffer System I

Stain:

O-dianisidine	10 mg
L-amino acid oxidase	10 mg
Peroxidase	20 mg
0.1 M phosphate buffer, *p*H 7.5	100 ml
0.1 M $MnCl_2 \cdot 4 H_2O$ (1.98 g/100 ml)	1 ml
Glycyl L-leucine	20 mg

Incubate at 37 C. Wash and fix. The stain also works well for bacterial strains.

Soak gel slice in HCl for 2 hr, thereby lowering the *p*H to about 2.0 (conversion of pepsinogen to pepsin). Pour off solution A and incubate gel in solution B until the gel develops a diffused light blue background, locally replaced by white zone of proteolytic activity. Wash and fix the gel.

Ribonuclease

Modification of the method of Ressler *et al.* (1966).

Buffer System I or XIV

Stain:

Yeast RNA	250 mg
Black K salt	100 mg
0.05 M acetate buffer, *p*H 5.0	100 ml
Acid phosphatase	10 mg

Incubate at 37 C until *blue* bands appear.

OXIDASES AND PEROXIDASES

Catalase

Buffer System II or V

Stain:

A. 3% H_2O_2	5 ml
0.1 M phosphate, pH 7.0	10 ml
0.06 M $Na_2S_2O_3 \cdot 5\ H_2O$	7 ml
H_2O	78 ml
B. 0.09 M KI	50 ml
H_2O	50 ml

Incubate gel in solution A at room temperature for 15 min. Pour off A and rinse with H_2O. Add solution B and let stand until gel is dark blue with white catalase bands.

Note: Mammalian tissue should be diluted 1 : 100 or more.

Ceruloplasmin

Method of Poulik and Bearn (1962).

Buffer System II

Stain Buffer: 0.1 M Na acetate, pH 5.7

Na acetate $\cdot$ 3 H_2O	12.3 g
0.1 M acetic acid	95.0 ml
H_2O to	1000 ml

Fixing Buffer: 0.1 M sodium acetate, pH 4.7 (see ribonuclease)

Stain:

Para-phenylene diamine hydrochloride	0.5 g
Stain buffer	100 ml

Incubate the gel in stain solution at 37 C for 1–3 hr. Decolorize gel by washing in 0.1 M Na acetate, pH 4.7.

Monoamine Oxidase

Adapted from Pearse (1961).

Buffer System V

Stain Buffer: 0.05 M phosphate

KH_2PO_4	8.71 g
0.5 M NaOH (20 g/liter)	46.8 ml
H_2O	1000 ml

Adjust pH with 0.5 M NaOH.

Stain:

A. 40% Na_2SO_4 (anhydrous, saturated)	50 ml
1.0 M NaOH	1 ml
0.2 M K_2HPO_4 (34.8 g/liter)	20 ml

B. 2-Hydroxy 3-naphthoic acid hydrazide	approx. 25 mg
C. 0.1 M tryptoamine hydrochloride (197 mg/ml)	10 ml
D. Fast blue B	200 mg
0.05 M phosphate buffer, *p*H 8.0	100 ml

Mix solution A, heat to 80–90 C, add B to make saturated solution. Cool, filter, and add reagent C. Incubate gel slice in this solution at 37 C for 1 hr or until pale yellow bands appear. Pour off the solution and replace by D. Keep in D for 10 min, pour off, add fixing solution. In about 30 min, violet bands against a blue background appear.

Peroxidase

Modification of the method of Graham *et al.* (1965).

Buffer System I or V

Stain:

A. 3-Amino 9-ethyl carbazole	50 mg
Dimethyl formamide	5 ml
0.05 M Na acetate, *p*H 5.0	92.5 ml
0.1 M $CaCl_2$	2 ml
3% H_2O_2	0.5 ml
B. Glycerine	50 ml
Distilled water	50 ml

Dissolve 3-amino 9-ethyl carbazole in dimethyl formamide, before adding other ingredients. Incubate gel in A in the cold room until the peroxidase area is stained reddish brown (30–60 min). Rinse gel with water and fix in solution B.

Tyrosinase

Modified method of Jolley and Mason (1966).

Buffer System II

Stain Buffer: 0.1 M phosphate, *p*H 7.0 (see pepsinogen)

Stain:

0.01 M catechol (110 mg/100 ml)	7.1 ml
0.01 M L-proline (115 mg/100 ml)	7.1 ml
0.1 M phosphate, *p*H 7.0	85.8 ml

Dissolve catechol and L-proline in phosphate buffer to make up to 0.01 M solution. Incubate gel in stain solution for 30–60 min, until bluish-orange bands appear. Fix the gel in glycerine.

DEHYDROGENASES

Alcohol Dehydrogenase (ADH)

Method of Shaw and Koen (1965).

Buffer System I or XVI

Stain:

NAD^+	50 mg
NBT	30 mg

PMS	2 mg
Ethanol (95%)	3 ml
0.1 M NaCN	5 ml
0.5 M tris–HCl, *p*H 7.1	15 ml
H_2O	77 ml

Incubate the gel at 37 C. Wash and fix.

D(−)3-Hydroxybutyrate Dehydrogenase (HBDH)

Method of Fottrell and O'Hora (1969).

Buffer System XVIII

Stain:

DL-hydroxybutyrate (IM)	10 ml
NAD	100 mg
PMS	2.5 mg
NBT	25 mg
0.5 M phosphate buffer, *p*H 7.4	25 ml
$MgCl_2$	10.2 mg
NaCl	575 mg
H_2O	65 ml

Incubate gel at 37 C until bands appear. Wash and fix.

Galactose 6-Phosphate Dehydrogenase (Gal-6PDH)

Buffer System I or III

Stain: Same as G6PD (glucose 6-phosphate dehydrogenase), but substitute 20 ml of 0.25 M Na galactose 6-phosphate for glucose 6-phosphate.

Substrate: 0.25 M Na gal 6-phosphate

Gal-6 P (barium salt)	5 g
1 M $(NH_4)_2SO_4$ (132.15 g/liter)	approx. 20 ml
0.1 M HCl	25 ml
1 M NaOH	to adjust *p*H
H_2O	50 ml

Dissolve galactose 6P (Ba) in HCl. Precipitate $BaSO_4$ by adding 20 ml $(NH_4)_2SO_4$. Centrifuge and to the supernatant add a few drops more of $(NH_4)_2SO_4$ to assure complete precipitation. Adjust to *p*H with 1 M NaOH. Make up to 50 ml with H_2O.

Glucose 6-Phosphate Dehydrogenase (G6PDH)

Buffer System III

Stain:

$NADP^+$	30 mg
NBT	20 mg
PMS	2 mg
0.5 M tris–HCl, *p*H 7.1	25 ml
Na_2 glucose $6P \cdot H_2O$	200 mg
H_2O	90 ml

Incubate gel at 37 C for 1 hr or until dark blue bands appear. Wash and fix.

Glutamate Dehydrogenase (GDH)

Buffer System I or III

Stain:

NAD^+	60 mg
NBT	30 mg
PMS	2 mg
0.5 M phosphate, *p*H 7.0	25 ml
Substrate (1 M Na glutamate, *p*H 7.0)	5 ml
H_2O	70 ml

Substrate: 1 M Na glutamate, *p*H 7.0

Na glutamate	16.9 g
0.5 M phosphate, *p*H 7.0	100 ml

Incubate gel at 37 C in the dark, until dark blue bands appear. Wash and fix. In case of bacterial glutamate dehydrogenase, $NADP^+$ is used instead of NAD^+ (see staining for bacterial extracts).

Glutathione Reductase

Method of Brewer (1966).

Buffer System VIII

Stain: The staining mixture is made up in 0.133 M tris, *p*H 7.6, and it contains:

Agar	1.0 g
EDTA	2.89 g
EDTA (reduced)	55.0 mg
Glutathione (oxidized)	3.02 g
5,5-Dithio-*bis*-(2-nitrobenzoic acid)	3.0 mg

The site of the enzyme activity is marked by a yellow color.

Glyceraldehyde 3-Phosphate Dehydrogenase (G3PDH)

Modification of method of Williams (1964).

Buffer System I or XVII

Substrate:

Na_4 fructose 1,6-diphosphate · 6 H_2O	545 mg
Aldolase	0.6 ml (100 units)
0.5 M tris–HCl, *p*H 7.1	4 ml
H_2O	6 ml

Mix and incubate substrate mixture at 37 C for 30 min.

Stain:

0.5 M tris–HCl, *p*H 7.1	20 ml
H_2O	70 ml
Substrate	10 ml
NAD^+	50 mg
NBT	30 mg

PMS	2 mg
Na_2HAsO_4	150 mg

Incubate gel at 37 C until blue bands appear. Wash and fix.

α-Glycerophosphate Dehydrogenase (αGPDH)

Buffer System I or III

Stain:

NAD^+	50 mg
NBT	30 mg
PMS	2 mg
1 M Na α-glycerophosphate (substrate), *p*H 7.0	10 ml
0.5 M tris–HCl, *p*H 7.1	15 ml
H_2O	70 ml
0.1 M NaCN	5 ml
Substrate: 1 M Na α-glycerophosphate, *p*H 7.0	21.6 g
H_2O	100 ml

Dissolve Na α-glycerophosphate in 50 ml of water. Adjust *p*H to 7.0 with 1 N HCl, make up to 100 ml. Incubate gel in stain mixture at 37 C until dark bands appear. Wash and fix.

Hexose 6-Dehydrogenase

Method of Shaw and Koen (1968*a*, *b*).

Buffer System III

Stain:

NBT	50 mg
NADP	50 mg
PMS	2 mg
Tris buffer, *p*H 6.8	10 ml
1 M galactose phosphate	5 ml
H_2O	85 ml

Incubate at 37 C until bands appear. Wash and fix.

Isocitrate Dehydrogenase (IDH)

Modification of the method of Henderson (1965).

Buffer System I or XIV

Stain:

$NADP^+$	20 mg
NBT	20 mg
PMS	3 mg
0.1 M Na_3 isocitrate · H_2O	5 ml
$MnCl_2 \cdot 4\ H_2O$	20 ml
H_2O	85 ml

Incubate gel at 37 C until blue bands appear. Wash and fix.

Lactate Dehydrogenase (LDH)

Buffer System I, VIII, or XIV

Stain: Same as α-glycerophosphate dehydrogenase, but substitute 1 M Na DL-lactate, *p*H 7.0.

1 M Na DL-lactate, *p*H 7.0:

85% DL-lactic acid	10.6 ml
1 M $Na_2CO_3 \cdot H_2O$	49 ml
H_2O to	100 ml

Keep flask cool during mixing.

Note: As optimum buffers for lactate dehydrogenase vary depending upon source of the enzymes, several buffer systems have been used. The following have been found to give the best results for the organisms and tissues indicated (Shaw and Koen, 1968*b*):

A. Mouse: buffer system XII
B. Human: buffer system XIII
C. Chicken: buffer system XIV
D. Beef muscle: buffer system XVII

Incubate at 37 C for 1 hr. Wash and fix.

Malate Dehydrogenase (MDH)

Method of Shaw and Koen (1964).

Buffer System I

Stain: Same as LDH, but substitute 1 M Na L-malate, *p*H 7.0, for lactate.

Substrate: 1 M Na L-malate, *p*H 7.0

L-malic acid	13.4 g
2 M $Na_2CO_3 \cdot H_2O$ (248 g/liter)	49 ml
H_2O	1000 ml

Make up same as Na lactate. Incubate at 37 C for 1 hr. Wash and fix.

6-Phosphogluconate Dehydrogenase (6PGD)

Buffer System III

Stain:

$NADP^+$	20 mg
NBT	25 mg
PMS	2 mg
Na_3 6-phosphogluconate	200 mg
(or 4 ml of 0.5 M Na_3 6-phosphogluconate)	
0.5 M tris–HCl, *p*H 7.1	10 ml
H_2O	90 ml

(If 4 ml of 0.5 M Na_3 6-phosphogluconate is used, add only 86 ml of H_2O.) Incubate gel at 37 C until bands appear. Wash and fix.

Retinol Dehydrogenase (RDH)

Method of Koen and Shaw (1966).

Buffer System VI

Stain Buffer: 0.1 M phosphate, *p*H 7.0 (see leucine aminopeptidase)

Substrate:

Retinol (vitamin A alcohol)	100 mg
Acetone	7 ml

Dissolve retinol in acetone immediately before use.

Stain:

NAD^+	66 mg
PMS	2 mg
NBT	35 mg
0.1 M phosphate buffer, *p*H 7.0	10 ml
Substrate	7 ml
H_2O	83 ml

Incubate gel at 37 C in dark, until dark blue bands appear. Wash and fix.

Sorbitol Dehydrogenase

Method of Lin *et al.* (1969).

Buffer System XIV

Stain:

Sorbitol	0.5 g
NAD	10 mg
MTT tetrazolium	15 mg
PMS	2 mg
0.05 M tris–HCl buffer, *p*H 8.0	100 ml

Incubate at 37 C. Wash and fix.

Xanthine Dehydrogenase

Buffer System III

Substrate: 1 M hypoxanthine

Hypoxanthine	13.6 g
1 M KOH	20 ml
H_2O	80 ml

Stain:

NAD^+	60 mg
NBT	30 mg
PMS	2 mg
1 M hypoxanthine	3 ml
0.5 M tris–HCl, *p*H 7.1	20 ml
H_2O	77 ml

Incubate at 37 C for 2 hr or until dark blue bands appear. Wash and fix.

MISCELLANEOUS

Adenylate Kinase (AK)

Modification of method of Fildes and Harris (1966).

Buffer System I

Stain:

NBT	20 mg
PMS	3 mg
$NADP^+$	25 mg
$MgCl_2 \cdot 6\ H_2O$	21 mg
Glucose	90 mg
0.5 M tris–HCl, *p*H 7.1	10 ml
H_2O	90 ml
ADP	20 mg
Hexokinase	0.08 ml (160 units)
G6PD	0.035 ml (80 units)

Incubate at 37 C until dark blue bands appear. Wash and fix.

Aromatic Amino Acid Transaminase

Method of Shaw and Baptist (1969).

Buffer System III

Substrate:

α-Ketoglutaric acid	1.0 g
H_2O	93 ml
1 M NaOH	13.8 ml

Adjust *p*H to 7.0.

Stain:

Pyridoxal phosphate	5 mg
L-tyrosine	100 mg[3]
NBT	30 mg
NAD^+	50 mg
PMS	2 mg
0.1 M phosphate, *p*H 7.0	50 ml
α-Ketoglutarate	10 ml
H_2O	30 ml
Glutamic dehydrogenase	0.2 ml[4] (*ca.* 16 E.U.)

Incubate at 37 C. Wash and fix.

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[3] Run a control without tyrosine.
[4] Add the enzyme just before use of the stain.

Creatine Kinase (CK)

Modification of method of Dawson *et al.* (1965).

Buffer System I

Stain:

Creatine phosphate	731 mg
ADP	75 mg
Glucose	90 mg
$MgCl_2 \cdot 6\ H_2O$	21 mg
NADP+	25 mg
PMS	3 mg
NBT	20 mg
0.5 M tris–HCl, *p*H 7.1	10 ml
H_2O	90 ml
Hexokinase	0.053 ml (160 units)
G6PD	0.08 ml (80 units)

Incubate gel at 37 C until bands appear. Wash and fix.

Fumarase

Buffer System III

Stain:

NAD^+	80 mg
NBT	30 mg
PMS	1 mg
K fumarate	770 mg
0.1 M phosphate, *p*H 7.1	20 ml
H_2O	80 ml
MDH	0.025 ml (200 units)

Incubate gel at 37 C until bands appear. Wash and fix.

Glucosephosphate Isomerase (GPI)

Method of Delorenzo and Ruddle (1969).

Buffer System XX

Stain:

0.1 M tris–HCl, *p*H 8	100 ml
$NADP^+$	10 mg
$MgCl_2$	80 mg
PMS	1 mg
MTT	10 mg
G6PD (Sigma)	5 μl
Fructose 6-phosphate	160 mg

Incubate gel at 37 C for about 1 hr. Wash and fix.

Glutamate-Oxaloacetate Transaminase (GOT)

Modification of the method of Schwartz *et al.* (1963).

Buffer System I

Stain:

L-aspartic acid	532 mg
α-Ketoglutaric acid	73 mg
Pyridoxal phosphate	50 mg
Fast violet B salt	200 mg
0.1 M phosphate buffer, *p*H 7.0	100 ml

Add fast violet B to rest of ingredients just before use. Incubate at 37 C until red-orange bands appear. Fix gel in glycerine. (Bands diffuse in the usual fixing solution.)

Hexokinase (HK)

Method of Eaton *et al.* (1966).

Buffer System I

Stain:

Glucose	90 mg
$MgCl_2 \cdot 6\ H_2O$	21 mg
ATP	25 mg
$NADP^+$	25 mg
PMS	3 mg
NBT	20 mg
G6PD	0.035 (80 units)
0.5 M tris–HCl, *p*H 7.1	10 ml
H_2O	90 ml

Incubate at 37 C. Wash and fix.

Phosphoglucomutase

Method of Spencer *et al.* (1964).

Buffer System I

Stain:

Na_2 glucose 1-phosphate $\cdot$ 4 H_2O	600 mg
$MgCl_2 \cdot 6\ H_2O$	200 mg
$NADP^+$	10 mg
Glucose 6-phosphate dehydrogenase	0.035 ml (80 units)
PMS	1 mg
NBT	20 mg
0.5 M tris–HCl, *p*H 7.1	10 ml
H_2O	90 ml

Incubate at 37 C in the dark until dark blue bands appear. Wash and fix.

Pyruvate Kinase (PK)

Buffer System IX

Stain Buffer: 0.433 M glycine buffer, *p*H 9.0

Glycine	32.5 g
1 M NaOH	65 ml
H_2O	1000 ml

Adjust *p*H with 1 M NaOH.

Stain:

Agar	375 mg
0.433 M glycine, *p*H 9.0	50 ml
Mg acetate · 4 H_2O	142.5 g
Na_3 phosphoenol pyruvate	14 mg
ADP	70 mg
LDH	40 units
NADH	56.5 mg

Dissolve agar in 40 ml of glycine buffer by heating to 100 C. Cool to 45 C. Mix remaining ingredients in 10 ml of glycine buffer and add to the cooled agar solution. Pour over gel slice and incubate at 37 C for 1–2 hr. View under ultraviolet light.

Thymidine Kinase

Method of Migeon *et al.* (1969).

Buffer System XIX

Stain:

ATP	5 mM (6.5 mg/2 ml)
$MgCl_2$ · 6 H_2O	5 mM (2.33 mg/2 ml)
Thymidine	57 μM (0.264 mg/2 ml)
0.1 M tris, *p*H 8.0	

Apply DEAE paper soaked with stain solution closely to the gel. Incubate as described for HGPRT. Remove DEAE paper from the gel and dry. Apply paper to X-ray film and expose for 10 days to 4 weeks. Dark bands appear in developed film.

Triosephosphate Isomerase (TPI)

Modification of method of Scopes (cited by Shaw and Koen, 1968*b*).

Buffer System I

Substrate: Dihydroxy acetone phosphate

1 M Na α-glycerophosphate	10 ml
1 M Na pyruvate	10 ml
NAD^+	50 mg
α-Glycerophosphate DH	0.16 ml (200 μg)
LDH	0.021 ml (200 μg)

0.2 M tris–HCl, *p*H 8.0	10 ml
H_2O	70 ml

Incubate at 37 C for 2 hr. Adjust *p*H to 2.0 with 1 N HCl to inactivate enzymes. Readjust *p*H to 7.0 with 1 M tris.

Stain:

NAD^+	60 mg
NBT	30 mg
PMS	2 mg
$Na_2HAsO_4 \cdot 12\ H_2O$	250 mg
Substrate solution	100 ml
Phosphoglyceraldehyde DH	1 ml (10 mg)

Incubate gel at 37 C until dark blue bands appear. Wash and fix.

BACTERIAL ENZYMES

Alanine Dehydrogenase

Method of Baptist *et al.* (1969).

Buffer System I or III

Stain:

NAD^+	50 mg
NBT	30 mg
PMS	2 mg
DL-alanine	100 mg
0.1 M phosphate, *p*H 7.0	100 ml

Incubate at 37 C until bands appear. Wash and fix.

L-Leucine Dehydrogenase

Method of Baptist *et al.* (1969).

Buffer System III

Stain: Same as alanine dehydrogenase, but substitute 50 mg of L-leucine for DL-alanine.

Peroxidase and Catalase

Method of Robinson (1966).

Buffer System II

Gel Wash: Take about 100 ml of electrode buffer and adjust the *p*H to 6.5 by adding 1 M HCl. Soak gel in this solution for 45 min at 0 C before staining.

Stain:

A. KI	2 g
H_2O	100 ml
Acetic acid	2 ml

B. H_2O 100 ml
3% H_2O_2 1 ml

Soak gel in solution A for 60 sec. Wash three times and add solution B. Incubate until peroxidase bands appear as dark blue bands and catalase bands appear white. Instead of gel wash, use 50% glycerine to fix the gel.

Pyrophosphatase

Buffer System II

Incubation Mixture:

$Na_4P_2O_7$	200 mg
$MnCl_2$	20 mg
0.5 M tris–HCl, *p*H 7.1	15 ml
H_2O	85 ml

Stain:

A. Molybdate

$(NH_4)6MO_7O_{24} \cdot 4\ H_2O$	2.50 g
H_2O	91.7 ml
Concentrated H_2SO_4	8.33 ml

B. Aminonaphtholsulfonic acid reagent
(Fisher "Gram-Pac" is used according to its direction.)

Incubate gel in incubation mixture at 37 C for 90 min. Wash and add 100 ml H_2O, 10 ml (A), and 4.0 ml (B). Blue bands are formed, but they are not permanent. If gel is kept 2–3 hr in gel wash, bands remain more permanent.

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