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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.

Table 1. Electrophoresis Buffer

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.

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.

[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)
$MgCl_2 \cdot 6\ H_2O$	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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