Letters to the Editors

phoroproliferative disorders15–21 and in human renal disease.11

AG MACIVER
BL MEPHAM
Faculty of Medicine,
South Laboratory Pathology Block,
Southampton General Hospital,
Tremena Road,
Southampton S09 4XY

References

2 Curran RC, Gregory J. Demonstration of immunoglobulin in cryostat and paraffin sections of human tonsil by immuno-
3 Denk H, Radaszkiewicz T, Weichert E. Pronase pretreatment of tissue sections enhances sensitivity of the unlabelled
4 Mepham BL. A study of the peroxidase-
antiperoxidase technique for the demon-
stration of intra-cellular immunoglobulin
in paraffin sections. M Phil thesis,
Southampton University Medical
School, 1980.
5 Sternberger LA. Some new developments
in immunocytometry. Mikroskopie
6 Petrali JP, Hinton DM, Moriarty GC,
Sternberger LA. The unlabelled antibody
enzyme method of immunocytometry.
Quantitative comparison of sensi-
tivities with and without peroxidase-
antiperoxidase complex. J Histochem
7 Taylor CR. The nature of Reed-Sternberg
cells and other malignant ‘reticulum’
8 Burns J. Background staining and sensi-
tivity of the unlabelled antibody-
enzyme (PAP) method. Comparison
with the peroxidase sandwich method
using formalin fixed paraffin embedded
9 Van Leeuwen FW. Immunoelectron
microscopic visualization of neuro-
hyphal hormones: Evaluation of some
tissue preparations and staining
procedures. J Histochem Cytochem
10 Mepham BL, Frater W, Mitchell BS.
The use of proteolytic enzymes to
improve immunoglobulin staining by
the PAP technique. Histochem J 1979;
11:345-58.
11 Maciver AG, Giddings J, Mepham BL.
Demonstration of extracellular immuno-
proteins in formalin-fixed renal biopsy
12 Averameas S, Tertyncz T. Peroxidase
labelled antibody and Fab conjugates
with enhanced intracellular penetration.
Immunochrom 1971;8:1175-9.
13 Goldman M. Fluorescent Antibody Meth-
14 Graham RC, Karnovsky MJ. The early
stages of absorption of injected horse-
radish peroxidase in the proximal
tubes of mouse kidney; ultrastructural
cytocytochemistry by a new technique.
15 Isaacs P, Wright DH. Intestinal
lymphoma: an immunohistological and histochemical study. J Pathol 1979;
129:179-90.
16 Jones DB, Castleden M, Smith JL,
Mepham BL, Wright DH. Immunoperoxidase
in angioimmunoblastic lymphoplasma.
17 Curran RC, Jones EL. Non-Hodgkin’s
lymphomas: an immunohistochemical
and pathological study. J Pathol 1979;
129:179-90.
18 Isaacs P. Middle East lymphoma and
alpha-chain disease. An immunohisto-
chemical study. Am J Surgical Pathol
19 Isaacs P. Immunohistochemical
demonstration of 'chain: a marker of B cell
malignancy. J Clin Pathol 1979;32:
802-7.
20 Isaacs P, Wright DH. Anomalous
staining patterns in immunohistologic
studies of malignant lymphoma. J
21 Isaacs P, Wright DH, Judd MA,
Mepham BL. Primary gastro-intestinal
lymphomas: A classification of 66

Dr Heyderman and her colleagues reply
as follows:

We are pleased to have the opportunity to reply to the letter by Dr Maciver and Mr Mepham.

We agree that we would all prefer a sensitive, reliable, and convenient immunoperoxidase method which does not entail
any special immunological purification procedures, but unfortunately this is not possible until affinity purified or mono-
clonal antisera are generally available, or manufacturers offer reliable absorbed
reagents as controls.

Absorption controls

Although validation of the reagents is
time-consuming and demanding, it is
essential and should precede any
investigation. The need for careful
screening and controls was recently
pointed out by Dr Walker in her paper

on the localisation of carcinoembryonic
antigen (CEA) in breast carcinoma.1

Work previously published using the
same antisera to CEA without absorp-
tion with normal cross-reacting antigen
(NCA; CEX) now needs to be re-evaluated.

‘Blocking’ controls

These were suggested by Goldman2 to
determine whether conjugation with
FITC changes specificity of an unlabelled
antibody. He does not recommend the
method for antisera raised in two different
species.

It has been shown3 that antisera raised
in different species may recognise different
antigenic determinants on the CEA
molecule. Some of the antisera are directed
to determinants on the carbohydrate
moiety and some against the protein
determinants. All are anti-CEA, but since
they recognise different parts of the
molecule one may not block the others.

It has also been shown that when rabbits are
immunised with a haptenised synthetic
trisaccharide, antisera to one, two, or
three of the sugars may result (Fournon,
personal communication). These antisera
may or may not block each other; all
would react with the parent antigen.

Finally, spleen cells from a single
immunised mouse when fused to myeloma
cells give rise to clones which may
recognise a different determinant on the original
immunogen.4

We have carried out three series of
experiments to investigate the use of
antisera to an antigen raised in two species
using one as a blocking control for the
other and have found the method un-
successful.

1 We were unable to block goat anti-CEA
binding by pretreatment with rabbit
anti-CEA (Dr Darcy).

2 We used antibodies to human placental
lactogen (HPL) raised in rabbits by Dr
Rosen and Ms Calvert and in swine
(Orion) on sections of placenta and a
secondary chorionicarcinoma. Application
of one failed to block binding of the
other when applied sequentially, though
the specificity of both antisera to HPL
was established by absorption experiments.

A second pair of antibodies used was
rabbit anti-IgG (Dakopatts) and goat
anti-IgG (Miles) applied to tonsil sections.
Again we failed to achieve complete
blocking.

3 We first demonstrated that we could
localise HPL in the placenta using a goat
anti-rabbit alkaline phosphatase conjugate
instead of a peroxidase conjugate. We then used double labelling to show that swine
anti-HPL (peroxidase indirect conjugate) did not block all the sites recognised by
the rabbit anti-HPL (visualised by the alkaline phosphatase conjugate).

Similar double labelling experiments with anti-immunoglobulins were difficult to
interpret because both antisera stained the stroma. However, our impression was
that blocking was incomplete. Even if blocking had been successful and both
antisera recognised the same determinant, the specificity of one of the antisera
would still require to be shown by loss of activity after absorption with IgG.

**PAP method**
Both the indirect and PAP methods are sensitive, reliable, and convenient. We use
the indirect method because it produces excellent results, and satisfactory
commercial peroxidase conjugates are available. Using the PAP technique we have
failed to show a consistent increase in sensitivity; an extra reagent is involved
so the number of variables is increased; it takes 45-60 minutes longer and is more
expensive.

There is no theoretical reason why the PAP method should increase specificity.
All second and subsequent antibodies are merely disclosing solutions, attaching only
to the immunoglobulin of the first antibody, and should have no antihuman
activity. The second antibody cannot (and should not) distinguish between the
specific and the contaminating antibodies which cause so-called 'background
staining'. If sensitivity is increased it must be to all the first antibodies.

Previous comparisons of the PAP and indirect methods have not used the same
second antibodies. A valid comparison would be a quantitative experiment
comparing the PAP method with a conjugate made with the same second
(unlabelled) antibody in similar antibody concentrations.

**Trypsinisation**
We regularly use antisera to many different antigens, including CEA, alphafetoprotein,
placental and pituitary hormones, HbAg, and epithelial membrane antigen, at
dilutions of 1:50 to 1:2000 and are able to demonstrate them satisfactorily without
enzyme pre-digestion. Improvement in staining for immunoglobulin after tryp-

**Conclusion**
Whether the indirect or PAP method is preferred or whether trypsinisation is
included or omitted remain matters for investigation and debate. In spite of the
problems of antigen purity and availability, absorption is the most reliable
control at the present time. Although we prefer affinity purified antisera, we use
several that are not so treated but produce good clean results, and all the
techniques we use are within the capacity of a routine department. In
immuno-

cytchemistry, as in histopathology in general, there is a need for quality control;
the validation of positive results is as important as their achievement.

**References**
1 Walker RA. Demonstration of carcino-

embryonic antigen in human breast


carcinomas by the immunoperoxidase
2 Goldman M. Fluorescent antibody methods.

New York and London: Academic Press,

1968;155-60.
3 Ormerod MG. Antigenic determinants of
carcinoembryonic antigen. Scand J

4 Kohler G, Milstein C. Continuous culture of

fused cells secreting antibody of

predefined specificity. Nature 1975;

5 Mason DY, Sammons RE. Alkaline

phosphatase and peroxidase for double

immunoenzymatic labelling of cellular

constituents. J Clin Pathol 1978;31:

454-60.
6 Heyderman E. The immunoperoxidase

method in histopathology: applications,
cell methods and controls. J Clin Pathol

7 Huang S-N, Minassian H, More JD.

Application of immunofluorescent stain-

ing on paraflin sections improved by

trypsin digestion. Lab Invest 1976;

35:383-90.
8 Darmady EM, Maciver AG. Renal

Pathology. London-Boston: Butter-

worths, 1980;516.

**Discrepancies in the weight of plastic vials**

When using single-pan analytical balances,

discrepancies of several milligrams in the

weight of plastic objects have been

observed. Occasionally a gradual drift in

the apparent weight was found to occur.

We found that weighing discrepancies

were worst with plastic vials or plastic

material when they were handled with

latex or vinyl disposable gloves. Even

minimal handling of the vial, such as that

involved in unscrewing and replacing the
cap while wearing gloves, was found to lead
to errors of the order of 1-5 mg. This

is illustrated in Table 1.

Of the various attempts to overcome

this problem, including spraying the

inside of the balance with antistatic fluid,

using earthed forceps to handle the vials,

placing earthed conducting foil over the

cap of the balance immediately below the

balance pan, and using an antistatic gun
to remove the charge on the vials, only

the last was found to give a measure of

success (Table 1). The discrepancies in

weight between columns I and III,

particularly in the case of the more sensi-

tive five-place balance—the Sartorius