

## SINGLE INCUBATION DOUBLE ESTERASE CYTOCHEMICAL REACTION USING A SINGLE COUPLING REAGENT

AINOON O MBBS, DCP, A J JABAMONEY, DipMLT and SK CHEONG MBBS, FRCP.

*Division of Haematology, Department of Pathology, Faculty of Medicine, University Kebangsaan Malaysia, Kuala Lumpur.*

### Summary

Most methods used in double esterase cytochemistry for the diagnosis and classification of acute myeloid leukaemias require double incubation and staining, using separate coupling reagents. We evaluated a method by Swirsky on our normal and abnormal blood and bone marrow smears where only a single incubation and the use of a single coupling reagent is required. Its short incubation period and its strong positive reaction for butyrate esterase in demonstrating cells of monocytic lineage gives it an advantage over the conventional double incubation technique.

Keywords: Cytochemistry, dual esterase, leukaemia.

### INTRODUCTION

Esterase cytochemistry plays an important role in the diagnosis and classification of acute myeloid leukaemias. The use of substrates which are specific for enzymes in granulocytic and monocytic cells allows the identification of acute leukaemias of these lineages.<sup>1</sup> Methods for combining two esterase stains on a single slide have enabled the simultaneous visualization of leukaemic cells exhibiting these substrate preferences. All methods previously described require staining of slides twice and using separate coupling reagents with each substrate – for example, fast blue BB with naphthol ASD chloracetate and fast garnet GBC with alpha-naphthyl butyrate.<sup>2</sup> In 1984, Swirsky described a single incubation double esterase technique where staining was carried out once using naphthol ASD chloracetate and alpha-naphthyl butyrate as substrates and a single coupling reagent (Fast blue BB).<sup>3</sup> Naphthol ASD chloracetate gave a bright blue reaction product predominantly in the neutrophils and their precursors. In contrast, alpha-naphthyl butyrate gave a dark brown reaction product predominantly in macrophages, monocytes and their precursors. This method has the advantages of firstly, shortening the staining time since only a single incubation with a single coupling reagent was required, and secondly, achieving a more intense positive reaction for butyral esterase compared to the traditional double method hence providing a better sensitivity for the identification of cells of monocytic lineage.

### MATERIALS AND METHODS

The single incubation double esterase method was performed on blood and bone marrow smears from 13 cases of acute myeloid leukaemia, 10 acute lymphoblastic leukaemia, 1 undifferentiated leukaemia, 1 chronic granulocytic leukaemia and 9 haematologically normal cases. The leukaemias were diagnosed by morphology and reactions for myeloperoxidase, PAS, acid phosphatase and Nasda (+/- NaF). Table 1 shows the acute leukaemia cases according to the FAB classification. The single incubation double esterase method was adapted from Swirsky's and is as follows:

#### Reagents

1. Buffered formalin/acetone (20 mg  $\text{Na}_2\text{HPO}_4$ , 100 mg  $\text{KH}_2\text{PO}_4$ , 30 ml distilled water, 45 ml acetone, 25 ml concentrated formalin).
2. Phosphate buffer 0.1 mol/L, 50 ml (5.5 ml  $\text{KH}_2\text{PO}_4$  mixed with 94.5 ml  $\text{KH}_2\text{PO}_4$ ) adjusted to pH 8.
3. Naphthol ASD chloracetate (Sigma No. 0758), 25 mg.
4. Alpha-naphthyl butyrate (Sigma No. 80000), 4 mg (4 ul).
5. Fast blue BB salt (Gurr/BDH No. 34177), 80 mg.

#### Method

1. Air dried smears were fixed in cold buffered formalin/acetone for 30 seconds.

2. They were washed briefly in distilled water and air dried.
3. Fast blue BB was mixed vigorously with phosphate buffer.
4. 1 ml of acetone was added to naphthol ASD chloracetate, agitated until dissolved and mixed with the **buffer/fast blue BB** salt.
5. Alpha-naphthyl butyrate was added to 1 ml of acetone, agitated until dispersed and mixed with the **buffer/Fast blue BB/naphthol AS-D chloracetate**.

**Precautions**

1. In step 3, it was important to shake the Fast blue BB salt vigorously to dissolve it completely, to avoid scums forming on the slides.
2. Throughout the staining, the Coplin jar should be kept in the dark.
3. Steps 4 and 5 should be carried out as rapidly as possible.

**RESULTS**

Granulocytes and their normal and

**TABLE 1**  
CASES OF ACUTE LEUKAEMIA EVALUATED ACCORDING TO FAB CLASSIFICATION

Lymphoblastic leukaemia	No.	Myeloblastic leukaemia	No.
L1	3	M1	1
L2	2	M2	3
L3	0	M3	2
T-ALL	2	M4	5
ALL (unclassified)	3	M5	2
<b>Total</b>	<b>10</b>	<b>Total</b>	<b>13</b>

6. The substrate solution was poured into a coplin jar, in which the fixed slides have been placed. They were allowed to incubate in the dark, at room temperature, for 20–30 mins.
7. After incubation, distilled water was run into the coplin jar until the colour turned from deep violet to clear.
8. Counterstaining was performed with **Mayer's Haematoxylin** for 5 mins.
9. The slides were then rinsed with distilled water, air dried and mounted with aqueous mounting medium.

leukaemic precursors stained bright blue. Monocytes and their normal and leukaemic precursors stained dark brown. Macrophages also stained dark brown. Lymphocytes, eosinophils, basophils, normoblasts and megakaryocytes were not positive for either of the **esterases**. The typical staining reactions of monoblasts and myeloblasts in a case of acute myelomonocytic leukaemia (M4 of FAB classification) are illustrated in Fig. 1. Table 2 summarises the reactions in the various types of acute leukaemias.

**TABLE 2**  
SINGLE INCUBATION DUAL ESTERASE REACTION IN THE DIFFERENT TYPES OF ACUTE LEUKAEMIAS (FAB CLASSIFICATION)

	M1	M2	M3	M4	M5	M6	L1	L2	L3	T-ALL
Chloracetate Esterase	+	+++	+++	+++	-	-	--	-	n.a.	-
Butyrate Esterase	--	-	-	+++	+++	-	-	-	n.a.	-

na = not available, + = positive reaction, - = negative reaction, +++ = strong positive reaction.

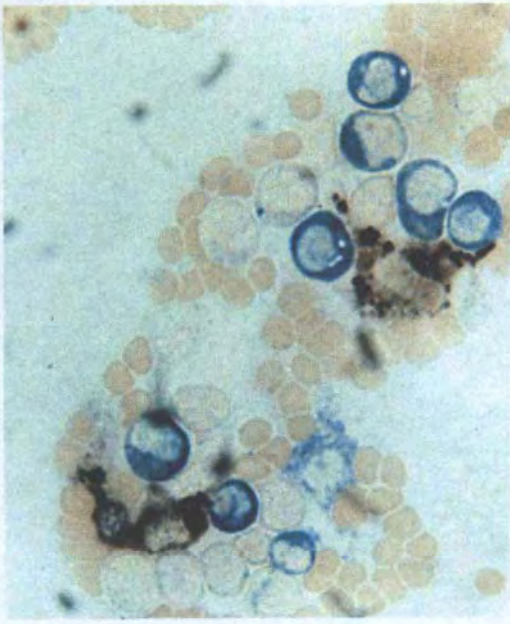


FIG. 1: Bone marrow stained with dual esterase in a case of acute myelomonocytic leukaemia M4 (X1000).

The two cases of T-ALL showed **negative** reactions for both chloracetate esterase and **butyrate** esterase. **T-ALL** was diagnosed by dot **positivity** for acid phosphatase and by **immunocytochemical** reactivity with **monoclonal anti-T (CD 2)**.

#### DISCUSSION

Performing two **esterase** reactions on a **single slide** to demonstrate **both** the **granulocytic** and **monocytic** components is a **useful procedure** in the diagnosis and classification of **acute leukaemias**. **Swirsky's** method of **single incubation** and **using a single**

coupling reagent has simplified the technique and shortened the staining time. In evaluating and adapting this **technique**, our **findings** are similar to that of Swirsky's where **granulocytes** and their precursors stained bright blue and monocytes and precursors stained dark brown. However we were not able to demonstrate the localised dark brown **positivity** in T-lymphoid cells described by Swirsky.

In our laboratory we have encountered problems with the traditional double esterase **stains** which require sequential incubation and staining with two different coupling reagents. One **problem** was the **failure** in achieving strong positive reactions for butyrate esterase. With Swirsky's modification, both reactions for chloracetate esterase and butyrate esterase appeared strong although **Swirsky** did not find any difference in **sensitivities** between his and the conventional method. However, care has to be taken especially with preparation of slides, **phosphate** buffer, incubation time and the amount of substrates and the coupling reagents used. We have found that slides **fixed in the cold buffered formalin/acetone** and phosphate buffer of pH 8.0 provided good preservation of cell morphology and maximal enzyme activity.

#### REFERENCES

1. Rozensajn L, Leibovich M, Shohan D, Epstein J. The esterase activity in **megaloblasts**, leukaemic and normal haemopoietic cells. *Br J Haematol* 1968; 14: 605-19.
2. Yam LT, Li CY, Crosby WH. **Cytochemical** identification of monocytes and **granulocytes**. *Am J Clin Pathol* 1971; 55: 283-90.
3. Swirsky DM. **Single** incubation double esterase **cytochemical** reaction using a single coupling reagent. Technical methods. *J Clin Pathol* 1984; 37: 1187-94.