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ABBREVIATIONS

^{32}P	phosphorus radioisotope
9-cis RA	9-cis retinoic acid
Ab	antibody
Ag	antigen
AML	acute myelogenous leukaemia
ATP	adenosine 5'-triphosphate
ATRA	all-trans retinoic acid
BSA	bovine serum albumin
<i>cdc</i>	cell division cycle
cDNA	complementary DNA
CO_2	carbon dioxide
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid

ELISA	enzyme linked immuno serum assay
ER	endoplasmic reticulum
FCS	fetal calf serum
GC	guanine-cytosine
HL-60	human acute promyelocytic leukaemic cells
Kb	kilobase
Kbp	kilobase pair
kDa	kilodalton
MOPS	3-(N-morpholino)-propanesulphonic acid
mRNA	messenger RNA
NaOH	sodium hydroxide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMA	phorbol-12-myristate 13-acetate
PTP-1B	protein tyrosine phosphatase-1B
RE	restriction endonuclease
RNA	ribonucleic acid
RNAse	ribonuclease
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
RT	reverse transcription

RTase	reverse transcriptase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	saline/ sodium citrate buffer
TBS	tris buffered saline
TE	tris EDTA
TEMED	N,N,N',N'-tetramethyl ethylene-diamine
UV	ultraviolet

CONFERENCE PROCEEDINGS

Bhoola R and Hammond K.D. Differentiation of HL60 cells and the expression of phosphoprotein phosphatases 1/2A and protein tyrosine phosphatase-1B. Proceedings of the 34th Annual Congress of the Federation of South African Societies of Pathology, Cape Town, July 1994

Bhoola R and Hammond K.D. The effect of All-trans retinoic acid on the expression of protein tyrosine phosphatase-1B in HL60 cells. Proceedings of the 35th Annual Congress of the Federation of South African Societies of Pathology, Bloemfontein, July 1995

Hammond K.D and Bhoola R. Expression of phosphoprotein phosphatase in proliferating and differentiating cells. The First UK-RSA Symposium on Cell Growth Control, Cape Town, January 1996

Bhoola R and Hammond K.D. Analysis of protein tyrosine phosphatase-1B messenger RNA and production of the recombinant protein. Proceedings of the 36th Annual Congress of the Federation of South African Societies of Pathology, Kruger Park Lodge, July 1996

PUBLICATIONS

Hammond K.D, Bhoola R, Bodalina U and Gilbert D (1998) Dynamic cells: temporal organizations and control of phosphorylation. Trends in Comparative Biochemistry and Physiology 4: 75-88.

Bhoola R, Hammond K. Modulation of the rhythmic patterns of expression of phosphoprotein phosphatases in human leukaemic cells. Cell Biol. Int. 2000, 24(8): 539-47.