Comparison of four rDNA primer sets (18S, 28S, ITS1, ITS2) for the molecular identification of yeasts and filamentous fungi of medical importance

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Invasive fungal infections are a significant cause of morbidity and mortality, especially in patients with an impaired immune system, including organ transplant patients following transplantation (e.g., single lung transplant patients with cystic fibrosis), patients with haematological malignancies (e.g., acute myeloid leukaemia) and patients with human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS).[1]

Use of conventional laboratory techniques for the isolation and identification of invasive fungal infection are less than optimal and hence molecular techniques are beginning to play an increasing role in their laboratory diagnosis. Correct identification of the yeast or filamentous fungal species is important for three reasons: i) correct clinical management of the mycological disease, ii) appropriate infection control of the patient/environment, and iii) correct epidemiological recording of the aetiological agent of the infectious process.

With the introduction of molecular techniques to many NHS trusts for the definitive identification of aetiological agents of infection, including fungi, there is considerable uncertainty about which primer sets are optimum for fungal identification, as many reports and primer sequences are available in the literature.

The aim of this study is to compare four sets of ribosomal DNA (rDNA) primers targeting the small subunit (18S rDNA), the large subunit (28S rDNA) and the short and the long interspacer (ITS) regions, as described in Figure 1, to discover which primer set would be most appropriate for use in an NHS diagnostic molecular service laboratory for the identification of yeasts and filamentous fungi of medical importance.

Nineteen fungal species were examined (Table 1). All fungal organisms were obtained from the Mycological Reference Laboratory, Department of Medical Microbiology, The Royal Group of Hospitals, Belfast, as either reference isolates or as highly characterised fungal isolates obtained from NEQAS/UKAS/CPA accreditation schemes. All isolates were cultured initially at 30°C for one week on Sabouraud dextrose agar (SDA; Oxoid CM0041, Oxoid, Basingstoke, UK).

All DNA isolation procedures were carried out in accordance with the DNA contamination management guidelines of Millar *et al.*[2] in a class II biological safety cabinet (MicroFlow, England). This was situated in a room physically separated from that used to set up nucleic acid amplification reaction mixes, and also from the 'post-PCR' room, in order to minimise contamination and the possibility of false-positive results.

Fungi were suspended in 1.5 mL Tris and were subjected to six cycles of freeze-thawing (liquid nitrogen/boiling). Genomic fungal DNA was further extracted from isolates using a high-purity polymerase chain reaction (PCR) template preparation kit (Roche, England), in accordance with the manufacturer's instructions. Extracted DNA was transferred to a clean tube and stored at –80°C prior to PCR.

A negative extraction control containing all reagents but no isolate was included with each batch of extractions. All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA, and from the amplification and post-PCR room, in order to minimise contamination.

For fungal identification, fungal DNA was amplified with four rDNA primer sets, as detailed in Table 2 and Figure 1. Reaction mixes (50 µL) contained 10 mmol/L Tris–HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 µmol (each) dATP, dCTP, dGTP and dTTP, 1.25 units *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin Elmer), 0.2 µmol (each) of the primer pairs and 4 µL DNA template containing approximately 5.0 ng DNA/mL extract.

Following a hot start, the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 96°C for 3 min, followed by 40 cycles of 96°C for 1 min, 55°C for 1min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. During each run, molecular grade water (Biowhittaker, Maryland, USA) was included randomly instead of DNA as a negative control, and *Candida albicans* DNA was included as a positive control.

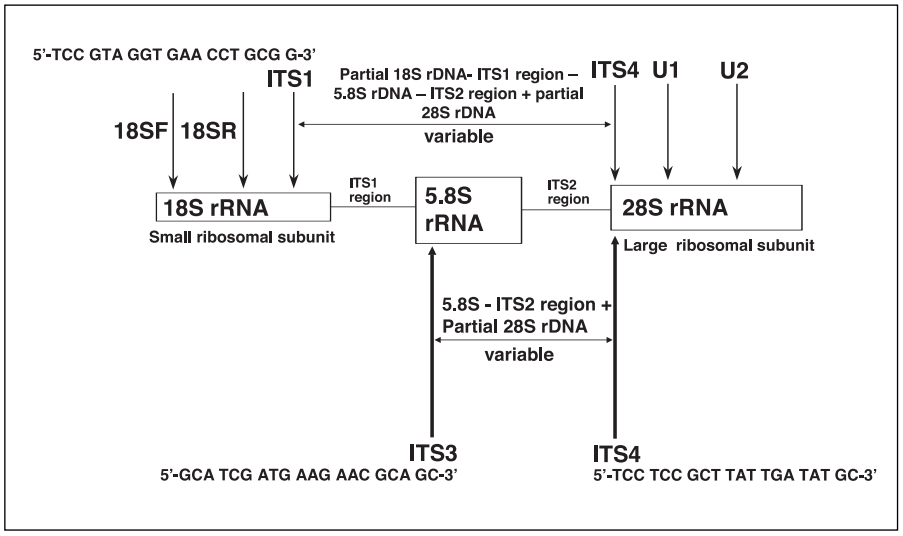

Fig. 1. Ribosomal DNA (rDNA) operon arrangement of the 28S, 18S and the interspacer (ITS) regions, with corresponding primer pair sequences in yeasts and filamentous fungi.

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Table 1. Comparison of a) 28S rDNA, b) 18S rDNA, c) long ITS region (ITS1/5.8S rDNA/ITS2) and d) short ITS region (5.8S rDNA/ITS2), employing 19 yeasts and filamentous fungi.

a

Organism	GenBank Acc. No.submitted	bp	Identity (%)	Identification
Acremonium alternatum	AY229884	180/196	91.8	*Acremonium alternatum* [U57349]
Alternaria alternata	AY679722	106/135	78.5	*Alternaria lonipes* [AY154684] *Alternaria mali* [AY154683] *Alternaria tenuissima* [AY154711]
Aspergillus flavus	AY661444	156/182	85.7	*Aspergillus flavus* [U26861]
Aspergillus fumigatus	AY230140	159/192	82.8	*Aspergillus fumigatus* [AY235006]
Aspergillus niger	AY669335	110/135	81.5	*Aspergillus niger* [AF109343] *Aspergillus nomius* [AF338613] *Aspergillus phoenicis* [AF433055]
Aspergillus terreus	AY247954	122/147	83.0	*Aspergilllus terreus* [U26877]
Candida albicans	AY230142	178/199	89.4	*Candida albicans* [L28817]
Candida dubliniensis	AY661442	173/201	86.1	*Candida dubliniensis* [AF405231]
Candida glabrata	AF441198	166/189	87.8	*Candida glabrata* [AF369519]
Candida guilliermondii	AY669332	167/188	88.8	*Pichia guilliermondii* [U26890]
Candida kefyr	AY233348	117/121	96.6	*Kluyveromyces marxianus* [AY363304] *Kluyveromyces lactis* [AY305672]
Candida krusei	AF417255	178/204	87.3	*Issatchenkia orientalis* [AY529502]
Candida lusitaniae		134/154	87.0	*Clavispora lusitaniae* [AY174089]
Candida parapsilosis	AF441199	158/189	83.6	*Candida parapsilosis* [U26898]
Candida tropicalis	AF441197	196/227	86.3	*Candida tropicalis* [L11349]
Chrysosporium keratinophilum	AY233350	128/149	85.9	*Aphanoascus fulvescens* [AF038357]
Cryptococcus neoformans	AY225573	194/224	86.6	*Filobasidiella neoformans* [AF356652]
Paecilomyces variotii	AY247956	135/168	80.4	*Paecilomyces* sp. [AF033395]
Scedosporium prolificans	AY230141	150/170	88.2	*Scedosporium prolificans* [AF027680]

b

Organism	GenBank Acc. No. submitted	bp	Identity (%)	Identification
Acremonium alternatum	AY229884	446/448	99.6	*Acremonium alternatum* [AY083232]
Alternaria alternata	AY679722	433/433	100	*Alternaria alternata* [U05194] *Alternaria brassicicola* [U05197] *Alternaria malorum* [AY251131] *Alternaria raphani* [U05199] *Clathrospora diplospora* [U43464] *Lewia infectoria* [U43465] *Ulocladium botrytis* [AF548105]
Aspergillus flavus	AY661444	357/359	99.4	*Aspergillus candidus* [AB008396] *Aspergillus clavatus* [AB008398] *Aspergillus flavus* [AF548060] *Aspergillus fumigatus* [AF548063] *Aspergillus glaucus* [AY083218] *Aspergillus oryzae* [D63698] *Aspergillus parasiticus* [D63699] *Aspergillus restrictus* [AB002079] *Aspergillus tamari* [AB106338] *Aspergillus sojae* [D63700] *Chaetosartorya cremea* [AB008399] *Edyuillia athecia* [AB002082] *Eupenicillium crustaceum* [D88324] *Eupenicillium javanicum* [U21298] *Eurotium herbariorum* [AF548072] *Eurotium repens* [AB002084] *Eurotium rubrum* [U00970] *Hemicarpenteles ornatus* [AB002067] *Hemicarpenteles paradoxus* [AB002080] *Neosartorya fischeri* [U21299] *Penicillium herquei* [AB086834] *Petromyces alliaceus* [AB002071]

b continued

Aspergillus fumigatus	AY230140	437/437	100	*Aspergillus candidus* [AB008396] *Aspergillus clavatus* [AB008398] *Aspergillus flavus* [AF548060] *Aspergillus fumigatus* [AF548063] *Aspergillus glaucus* [AY083218] *Aspergillus oryzae* [D63698] *Aspergillus parasiticus* [D63699] *Aspergillus restrictus* [AB002079] *Aspergillus sojae* [D63700] *Aspergillus tamarii* [AF516140] *Chaetosartorya cremea* [AB008399] *Eupenicillium javanicum* [U21298] *Eurotium herbariorum* [AF548072] *Eurotium repens* [AB002084] *Eurotium rebrum* [U00970] *Hemicarpenteles ornatus* [AB002067] *Neosartorya fischeri* [U21299] *Penicillium herquei* [AB086834] *Petromyces alliaceus* [AB002071]
Aspergillus niger	AY669335	350/350	100	*Aspergillus awamori* [D63695] *Aspergillus niger* [AF548064]
Aspergillus terreus	AY247954	438/438	100	*Aspergillus terreus* [AF516138] *Fennellia flavipes* [AB002062]
Candida albicans	AY230142	414/414	100	*Candida albicans* [M60302]
Candida dubliniensis	AY661442	414/414	100	*Candida dubliniensis* [AY497766]
Candida glabrata	AF441198	433/434	99.8	*Candida glabrata* [AY046237]
Candida guilliermondii	AY669332	441/441	100	*Candia guilliermondii* [AJ508270] *Candida xestobii* [AY227715] *Pichia guilliermondii* [AY249515]
Candida kefyr	AY233348			
Candida krusei	AF417255	414/414	100	*Candida glycerinogenes* [AY584809] *Candida krusei* [M55528] *Issatchenkia orientalis* [AY218894] *Pichia norvegensis* [AF201302]
Candida lusitaniae		415/415	100	*Clavispora lusitaniae* [AY497762]
Candida parapsilosis	AF441199	408/408	100	*Candida parapsilosis* [AY055857]
Candida tropicalis	AF441197	390/391	99.7	*Candida tropicalis* [M60308]
Chrysosporium keratinophilum	AY233350	434/435 435/436	99.8 99.8	*Coccidioides immitis* [X58571] *Aphanoascus fulvescens* [AJ315172]
Cryptococcus neoformans	AY225573	443/443	100	*Filobasidiella neoformans* [AY083224]
Paecilomyces variotii	AY247956	155/159	97.5	*Aspergillus* spp. (n=20) *Paecilomyces varotii* [Y13996] Other genera (n=18)
Scedosporium prolificans	AY230141	430/430	100%	*Lomentospora prolificans* [U43910] *Graphium tectonae* [U43907] *Graphium fructicola* [AB007659]

c

Organism	GenBank Acc. No. submitted	bp	Identity (%)	Identification
Acremonium alternatum	AY229884	304/306	99.3	*Acremonium strictum* [AY138844]
Alternaria alternata	AY679722	419/419	100	*Alternaria abutilonis* [AF314578] *Alternaria alternata* [AF455539] *Alternaria arborescens* [AF397244] *Alternaria citri* [AF314579] *Alternaria compacta* [AF314573] *Alternaria gaisen* [AF314574] *Alternaria lini* [AY17071] *Alternaria longipes* [AY154684] *Alternaria mali* [AY154683] *Alternaria pomicola* [AF314583] *Alternaria tenuissima* [AY154712]

c continued

Aspergillus flavus	AY661444	447/448	99.8	*Aspergillus flavus* [AF078893] *Aspergillus oryzae* [AB000533]]
Aspergillus fumigatus	AY230140	395/395	100	*Aspergillus fumigatus* [AY373851]
Aspergillus niger	AY669335	362/362	100	*Aspergillus awamori* [AY373840] *Aspergillus foetidus* [AY373850] *Aspergillus niger* [AY373852]
Aspergillus terreus	AY247954	485/486	99.8	*Aspergillus terreus* [AF455426]
Candida albicans	AY230142	463/463	100	*Candida albicans* [AF455457]
Candida dubliniensis	AY661442	422/422	100	*Candida dubliniensis* [AB049123]
Candida glabrata	AF441198	362/363	99.7	*Candida glabrata* [AY139784]
Candida guilliermondii	AY669332	413/413	100	*Pichia guillermondii* [AF455495]
Candida kefyr	AY233348	415/415	100	*Kluyveromyces marxianus* [AY046214]
Candida krusei	AF417255	413/415	99.3	*Issatchenkia orientalis* [L47113]
Candida lusitaniae		251/251	100	*Clavispora lusitaniae* [AY321473]
Candida parapsilosis	AF441199	382/383	99.7	*Candida parapsilosis* [L47109]
Candida tropicalis	AF441197			
Chrysosporium keratinophilum	AY233350	458/458	100	*Chrysosporium keratinophilum* [AJ131681]
Cryptococcus neoformans	AY225573	392/392	100	*Filobasidiella neoformans* [AF356652]
Paecilomyces variotii	AY247956	390/390	100	*Paecilomyces varotii* [AF455416]
Scedosporium prolificans	AY230141	426/428	99.5	*Scedosporium prolificans* [AF022485]

d

Organism	GenBank Acc. No. submitted	bp	Identity (%)	Identification
Acremonium alternatum	AY229884	298/298	100	*Acremonium strictum* [AY138844]
Alternaria alternata	AY679722	289/289	100	*Alternaria abutilonis* [AF314578] *Alternaria alternata* [AF455539] *Alternaria arborescens* [AF397244] *Alternaria citri* [AF314579] *Alternaria compacta* [AF314573] *Alternaria gaisen* [AF314574] *Alternaria longipes* [AY154684] *Alternaria mali* [AY154683] *Alternaria pomicola* [AF314583] *Alternaria tenuissima* [AY154712]
Aspergillus flavus	AY661444	290/290	100	*Aspergillus flavus* [AY373848] *Aspergillus oryzae* [AY373857]
Aspergillus fumigatus	AY230140	301/301	100	*Aspergillus fumigatus* [AF138288]
Aspergillus niger	AY669335	285/285	100	*Aspergillus awamori* [AY373840] *Aspergillus foetidus* [AY373850] *Aspergillus niger* [AY373852]
Aspergillus terreus	AY247954	296/296	100	*Aspergillus terreus* [AJ413985]
Candida albicans	AY230142	288/288	100	*Candida albicans* [AY196001]
Candida dubliniensis	AY661442	293/293	100	*Candida dubliniensis* [AF405231]
Candida glabrata	AF441198	343/344	99.7	*Candida glabrata* [AY139784]
Candida guilliermondii	AY669332	318/318	100	*Pichia guillermondii* [AF455495]
Candida kefyr	AY233348	369/369	100	*Kluyveromyces marxianus* (anamorph: *Candida kefy*) [AF543841]
Candida krusei	AF417255	307/307	100	*Issatchenkia orientalis* [L47113]
Candida lusitaniae		207/207	100	*Clavispora lusitaniae* [AF218970] *Clavispora imtechensis* [AY174102]
Candida parapsilosis	AF441199	269/270	99.6	*Candida parapsilosis* [L47109]
Candida tropicalis	AF441197	295/295	100	*Candida tropicalis* [AF218992]
Chrysosporium keratinophilum	AY233350	230/230	100	*Chrysosporium keratinophilum* [AJ131681]
Cryptococcus neoformans	AY225573	310/310	100	*Filobasidiella neoformans* [AF356652]
Paecilomyces variotii	AY247956	316/316	100	*Paecilomyces variotii* [AF033395]
Scedosporium prolificans	AY230141	270/272	99.3	*Scedosporium prolificans* [AF022485]

Table 2. Ribosomal DNA primer pairs and corresponding nucleotide sequences employed in this study.

Primer	Sequence 5'.....3'	Target gene	Region amplified	Reference
U1	GTG AAA TTG TTG AAA GGG AA	28S rRNA	28S rRNA	3
U2	GAC TCC TTG GTC CGT GTT	28S rRNA		
18SF	ATT GGA GGG CAA GTC TGG TG	18S rRNA	18S rRNA	4
18SR	CCG ATC CCT AGT CGG CAT AG	18S rRNA		
ITS1	TCC GTA GGT GAA CCT GCG G	18S rRNA	18S/ITS1/5.8S/	5
ITS4	TCC TCC GCT TAT TGA TAT GC	28S rRNA	ITS2/28S	
ITS3	GCA TCG ATG AAG AAC GCA GC	5.8S rRNA	5.8S/ITS2/28S	5
ITS4	TCC TCC GCT TAT TGA TAT GC	28S rRNA		

The specificity of all rDNA primer sets were challenged, as described above, with the individual introduction of genomic DNA from bacteria (including *Bartonella quintana*, *Chlamydia pneumoniae*, *Coxiella burnetii*, *Escherichia coli*, *Staphylococcus aureus* and *Steptococcus pneumoniae*) and from human lymphocytic tissue.

Following amplification, 8-µL samples were removed from each reaction mixture and examined by electrophoresis (100 V, 45 min) in gels composed of 2% (w/v) agarose (Gibco, Paisley, Scotland, UK) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]) and stained with ethidium bromide (5 µg/100 mL). Gels were visualised under ultraviolet (UV) illumination with a gel image analysis system (UVP Products, England) and all images were archived as digital graphic bitmap files (*.bmp).

Amplicons were identified by direct sequencing techniques. All amplicons were purified using a QIAquick PCR purification kit (Qiagen, UK) eluted in Tris–HCl (10 mmol/L [pH 8.5]) prior to sequencing, particularly to remove dNTPS, polymerases, salts and primers. For each primer pair, sequencing was performed in both the forward and reverse directions. In both cases, the sequencing primer was labelled with Cy-5′. This was used in conjunction with the ALF Express II (Amersham-Pharmacia, Buckinghamshire, England), using the Thermo Sequenase fluorescence-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, UK; RPN 2438) at 96°C for 1 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 5 sec, followed by a 4°C hold.

Resulting sequences were confirmed by chromatogram analysis and confirmed sequences were compared with those stored in the GenBank using the BLASTn alignment software (www.blast.genome.ad.jp/). Sequence homology identity was determined in accordance with the criteria described previously.[6] Subsequently, all sequences were submitted to GenBank and a corresponding accession number was allocated (Table 1).

All 19 fungi examined generated a PCR amplicon for each primer pair tested, but no PCR amplification products were detected when the method was challenged with bacterial or human genomic DNA (data not shown), demonstrating that these primers are specific for the range of fungal organisms tested.

Subsequent BLASTn search of resulting sequences of the four rDNA gene loci examined for each of the 19 fungal organisms is shown in Table 1. Subsequent analysis of these data in terms of i) correct identification to genus level, ii) correct identification to species level, iii) mean percentage identification and iv) percentage specificity (i.e., ability to differentiate target organism from neighbouring species) is shown in Table 3.

Use of the large subunit (28S rDNA) gave the poorest sequencing results (86.2%), with multiple ambiguities (*n*) in the resulting sequencing chromatograms, which take extra time with their analyses. However, this primer pair (U1/U2) yielded the highest specificity (i.e., this primer pair was best able to differentiate the target organism from close phylogenetic neighbours [species]). In contrast, use of the small subunit (18S rDNA) gave almost perfect sequencing, with very few sequencing ambiguities, and was easy to analyse.

The significant drawback to the use of this primer pair (18SF/18SR) was that it targeted highly conserved regions; thus, it was very difficult to speciate the target organism, as it yielded several equally possible alternatives to the target organism, due to a high degree of rDNA conservation among closely related species.

The short and long ITS regions displayed several advantages over the 18S and 28S rDNA gene loci in terms of sequencing quality and the ability to identify the target

Table 3. Analyses of sequencing ability of four rDNA primer pairs for the correct identification of yeasts and filamentous fungi of medical importance.

Parameter	28S rDNA	18S rDNA	Long ITS region (ITS1/5.8S/ITS2)	Short ITS region (5.8S/ITS2)
Correct identification to genus level	94.7%	94.7%	100%	100%
Correct identification to species level	94.7%	94.7%	89.4%	94.7%
Mean identification	86.2%	99.8%	94.6%	99.9%
Specificity	84.2%	36.8%	73.7%	63.2%

organism correctly. The long ITS had an advantage over the short ITS region, as it was more specific, thus allowing more reliable speciation of target organisms.

Health service laboratories adopting molecular rDNA methods for the correct identification of medically important fungi from culture are faced with the dilemma of choosing which rDNA region, and hence which primer pair or pairs, to use from the numerous options reported in the literature. The present study demonstrates that each primer pair has its own sets of advantages and disadvantages in terms of the criteria described above (Table 3).

Overall, the results of this study support the use in tandem of both the short and the long ITS rDNA regions for the purposes of molecular (rDNA) identification and reconfirmation of fungi of medical importance.

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Use of heat provides a fast and efficient way to undertake melanin bleaching with dilute hydrogen peroxide

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Heavily pigmented melanocytic lesions can often be difficult to interpret especially when attempting to define nuclear atypia and therefore substantiating a benign or malignant diagnosis. The presence of melanin pigment poses two fundamental problems. First, it has a direct physical masking effect on antigen–antibody interactions, due to the intracellular nature of the pigment. Second, 3,3-diaminobenzidene (DAB), the most widely employed chromogen for demonstrating antigen/antibody reactions, is also brown in colour and therefore can be difficult to distinguish from melanin pigment.

Table 1. Antibodies used to assess the effects of melanin bleaching using dilute hydrogen peroxide.

Antibody	Source	Dilution	Pretreatment
S100 protein	Dako	1:4000	Trypsin
HMB 45	Launch	1:40	Microwave
NKIC3	Launch	1:40	Microwave
Melan A	Launch	1:30	Microwave
CD3	Vision Biosystems	1:100	Microwave
CD20	Dako	1:500	Microwave
CD68	Dako	1:100	Trypsin
CD34	Vision Biosystems	1:25	None
CD45	Dako	1:50	Microwave
CD31	Dako	1:10	Trypsin
SMA	Dako	1:250	None

Dako, Denmark House, Angel Drove, Ely, Cambridge, UK.
Launch Diagnostics, Ash House, Ash Road, New Ash Green, Longfield, Kent.
Vision Biosystems (Europe). Balliol Business Park West, Benton Lane, Newcastle upon Tyne.

Traditional techniques have used coloured chromogens such as alkaline phosphatase (red)[1] or have employed tinctorial techniques to stain melanin pigment a different colour from the antigen–antibody final reaction product (e.g., azure B).[2] These methods have merit in certain circumstances, but, in the author's experience with cutaneous and metastatic deposits of heavily pigmented lesions, they do not always enable accurate assessments of the exact localisation of antigen–antibody interactions (e.g., membrane or cytoplasmic labelling profiles). This problem may be exacerbated if the antigenic epitope is co-expressed on cell membranes and in the cytoplasm (e.g., S100 protein).

The two widely used bleaching procedures, permanganate/oxalate and dilute hydrogen peroxide (H_2O_2), have advantages and disadvantages.[3–7] In the author's experience, the two most significant factors are bleaching time and the range of antibodies employed. Permanganate/oxalate has a clear advantage, as bleaching can be achieved in most cases within an hour and can be incorporated easily in conventional daily immunostaining protocols. In contrast, the H_2O_2 method requires 24 hours.

However, the range of antibodies that can be used following these two bleaching procedures varies. The H_2O_2 method allows extensive application of antibodies, whereas permanganate/oxalate permits a useful but restricted range.[8]

The use of heat-mediated antigen retrieval techniques in immunocytochemistry is now well established, and whether or not such methods can be utilised to increase the speed of

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