



UNIVERSITI PUTRA MALAYSIA

**DEVELOPMENT OF NOVEL PRIMERS AND PROBES BASED ON ITS 2 REGION
USING NESTED-PCR AND DNA HYBRIDIZATION ARRAY FOR *CANDIDA*
SPECIES IDENTIFICATION**

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REGION USING NESTED-PCR AND DNA HYBRIDIZATION ARRAY
FOR *CANDIDA* SPECIES IDENTIFICATION**

By

DAVID CHIENG CHING SOO

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Master of Science**

February 2007



Specially dedicated to,

My beloved grandmother, parent, sister, Tsuey Peng, and all my family members

For their invaluable love, understanding, encouragement and patience

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment
of the requirement for the degree of Master of Science

**DEVELOPMENT OF NOVEL PRIMERS AND PROBES BASED ON ITS 2
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Chairman: Chong Pei Pei, PhD

Faculty: Faculty of Medicine and Health Sciences

Sensitivity, specificity, simplicity, speed, and economy fairly well describe the desirable attributes of any clinical diagnostic test. In the mycology laboratory, the detection and differentiation of *Candida* species usually rely on the morphological, physiological, and biochemical characteristics which are complex, frequently slow to appear, sometimes vary within a species, and usually require significant experience to evaluate.

In this study, novel primers and probes had been developed for the identification of 8 *Candida* species using nested-PCR and DNA hybridization array by focusing on a

single parameter. This parameter is the identification of nucleotide sequence from ITS 2 region that is known to be unique for the *Candida* species. These distinctive sequences were used to design and develop *Candida* species-specific primers and probes for nested-PCR and DNA hybridization array, respectively. For nested-PCR, the universal fungal primers, ITS 3 and ITS 4 were used to amplify the portion of the 5.8S and 28S rDNA, and the ITS 2 region of *Candida* species. The amplicons were used as template together with developed species-specific primers in the subsequent amplification. The results were analyzed using agarose gel electrophoresis. In addition, species-specific probes were used to hybridize to the biotinylated *Candida* species ITS 2 amplicons via DNA hybridization array. The amplicons were detected colorimetrically in strip format.

The sensitivity and specificity of the *Candida* species-specific primers and probes were evaluated. The 8 *Candida* ATCC strains and 24 clinical isolates did not exhibit cross-priming and cross-hybridization with the species-specific primers and probes in both assays and all the *Candida* species were correctly identified. In simulated clinical specimens, the sensitivity of the nested-PCR for *Candida* species detection was 10 cells/mL. However, the detection limit for both PCR using species-specific primers and DNA hybridization array using species-specific probes for the detection of *Candida* culture DNA was 1-10 cells/mL.

Additionally, a preliminary study was done for the screening of 40 healthy donors' sera using the real-time PCR for the detection of *Candida albicans* using species-specific primers, CAL1 and CAL3. The melting curve was used for the analyses of the results. 3 out of 40 samples were found to be positive for *Candida albicans*. It is suggested that the real-time PCR may not be able to distinguish the individuals who are colonized from those who are infected.

In conclusion, the nested-PCR and DNA hybridization array using the developed species-specific primers and probes, respectively, in this study are robust, sensitive and can be easily integrated into a clinical diagnostic laboratory with the potential for same-day diagnosis of *Candida* infection. In addition, the simultaneous differentiation of *Candida* species via DNA hybridization array allows faster and simpler diagnosis compare to nested-PCR. For real-time PCR screening of the healthy donors' sera, further evaluation needs to be done to determine a threshold as standard guideline to detect the infectious and colonized *Candida albicans* and non-*albicans Candida* species.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PERKEMBANGAN PRIMER DAN PROBE YANG NOVEL BERDASARKAN
BAHAGIAN ITS 2 MELALUI NESTED-PCR DAN DNA HYBRIDIZATION
ARRAY UNTUK PENGESANAN SPESIES *CANDIDA***

Oleh

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Sensitif, spesifik, ringkas, cepat, dan murah disifatkan sebagai ciri-ciri yang diingini bagi setiap ujian diagnostik di makmal. Dalam makmal mikologi, pengesanan dan pembezaan bagi spesies *Candida* kebiasanya bergantung kepada penampilan sifat secara morfologi dan fisiologi serta ciri-ciri biokimia yang kompleks yang muncul lewat. Kadangkala ianya berbeza sesama spesies dan pengalaman pegawai bagi menilai keputusan amat diperlukan dalam diagnosis tersebut.

Dalam projek ini, primer-primer dan probe-probe baru telah dibangunkan bagi pembezaan antara 8 spesies *Candida* melalui dua kaedah molekul, iaitu

“nested-PCR” dan “DNA hybridization array” dengan menumpu kepada satu parameter tunggal. Parameter tersebut adalah pengesanan jujukan nukleotida daripada bahagian ITS 2 yang unik untuk spesies *Candida*. Jujukan nukleotida tersebut ini telah digunakan untuk mereka dan menghasilkan primer dan probe yang spesifik bagi spesies *Candida* untuk digunakan dalam “nested-PCR” dan “DNA hybridization array”. Bagi “nested-PCR”, primer fungi yang universal iaitu ITS 3 dan ITS 4 telah digunakan dalam amplifikasi sebahagian daripada 5.8S dan 28S rDNA, dan seluruh bahagian ITS 2 spesies *Candida*. Hasil PCR ini telah digunakan sebagai templat bersama dengan primer spesies *Candida* yang spesifik untuk amplifikasi berikutnya. Hasil amplifikasi tersebut dianalisis menerusi elektroforesis gel agarosa. Bagi “DNA hybridiazation array”, probe spesies *Candida* yang spesifik digunakan dihibridkan dengan hasil amplifikasi bahagian ITS 2 spesies *Candida* yang dilabel dengan biotin. Ianya dikesan secara kolorimetri pada strip yang disediakan.

Sensitiviti dan kespesifikan primer dan probe yang spesifik kepada spesies *Candida* telah dinilai. 8 strain ATCC dan 24 sampel spesies *Candida* tidak menunjukkan sebarang “cross-priming” dan “cross-hybridization” terhadap primer dan probe yang diuji dalam kedua-dua kaedah tersebut. Semua sampel dan strain *Candida* telah dikenalpasti dengan tepat. Dalam spesimen-spesimen klinikal yang tersimulasi, sensitiviti bagi “nested-PCR” dalam pengesanan spesies *Candida* ialah 10 sel/ml. Walau bagaimanapun, had pengesanan bagi “PCR” dan “DNA hybridization array”

dengan menggunakan primer dan probe, masing-masing, dalam pengesanan DNA spesies *Candida* dari kultur ialah 1-10 sel/ml.

Sebagai tambahan, satu kajian awal telah dijalankan untuk penyaringan sampel-sampel serum daripada 40 penderma darah yang sihat melalui “real-time PCR” bagi pengesanan terhadap spesies *Candida albicans* menggunakan primer yang spesifik terhadap *Candida albicans*, iaitu *CAL 1* dan *CAL 3*. Hasilnya dianalisa menggunakan “melting curve”. 3 daripada 40 sampel tersebut didapati positif untuk *Candida albicans*. Keputusan tersebut mencadangkan bahawa “real-time PCR” berkemungkinan tidak berupaya bagi membezakan individu yang mempunyai *Candida albicans* yang bersifat komensal daripada individu yang terjangkit.

Sebagai kesimpulan, “nested-PCR” dan “DNA hybridization array” yang digunakan dalam projek ini adalah sensitif dan spesifik dengan menggunakan primer dan probe yang spesies-spesifik masing-masing. Ianya amat berpotensi untuk tujuan diagnosis di dalam makmal klinikal kerana keputusan pengesanan spesies *Candida* boleh dihasilkan dalam masa satu hari. Di samping itu, pembezaan spesies *Candida* secara setara melalui “DNA hybridization array” boleh menghasilkan keputusan diagnostik dengan cepat dan mudah. Bagi penyaringan sampel serum daripada penderma-penderma sihat melalui “real-time PCR”, ianya perlu dilakukan terhadap sejumlah sampel yang besar bagi menentukan satu ambang sebagai garis panduan yang standard untuk mambezakan sama ada seseorang itu mempunyai *Candida*

albicans yang bersifat komensal atau terjangkit atau spesies-spesies *Candida* yang lain.

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I certify that an Examination Committee has met on 9 February 2007 to conduct the final examination of David Chieng Ching Soo on his Master of Science thesis entitled “Development of Novel Primers and Probes Based on ITS 2 Region Using Nested-PCR and DNA Hybridization Array for *Candida* Species Identification” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

DAVID CHIENG CHING SOO

Date: 4 APRIL 2007

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	x
APPROVAL	xii
DECLARATION	xiv
LIST OF TABLES	xix
LIST OF FIGURES	xx
LIST OF ABBREVIATIONS	xxiv
CHAPTER	
1 INTRODUCTION	
Background of the study	1
Objectives	4
 2 LITERATURE REVIEW	6
<i>Candida</i> species	6
Candidiasis	12
Prevalence of <i>Candida</i> species	13
Laboratory diagnosis of invasive candidiasis	17
Microscopic identification	19
Biochemical tests for species differentiation	
Commercial yeast identification systems	20
Molecular methods for detection and identification	21
Hybridization	
The Internal Transcribed Spacer (ITS) region	26
Polymerase Chain Reaction (PCR) and its mechanism	29
Primer Designing Based on PCR	31
Primer Length	
Melting temperature (T_m)	33
Specificity	35
Complementary primer sequences	37
G/C content and polypyrimidine (T, C) or polypurine (A, G)	38
stretches	39
3'-end sequence	40
DNA hybridization array	
The principle	40
Characteristics of probe and target nucleic acids	41
Probe form	41
Probe size	41
Probe specificity	

Choice of target nucleic acids	42
Probe labeling	42
Real-time PCR	43
Specific detection methods	44
Taqman probes	44
Molecular Beacons	45
Scorpion primers	46
Non-specific detection methods	49
Real-time PCR as a diagnostic assay	50
	51
	52
	53
	54
3 MATERIALS AND METHODS	59
Sequence comparison of the <i>Candida</i> ITS 2 region	
Designing species-specific primers and probes for the <i>Candida</i> species	59
Culture of the <i>Candida</i> species	59
Preparation of simulated clinical sera	61
DNA extraction for <i>Candida</i> species from culture	61
DNA extraction for <i>Candida</i> species from serum	62
PCR amplification using ITS 3 and ITS 4 primers	63
PCR amplification using species-specific primers	64
The specificity test using species-specific primers	65
The sensitivity test using species-specific primers	66
Agarose gel electrophoresis	67
PCR product purification for sequencing	67
PCR for the amplification of the biotinylated PCR product	68
Agarose gel electrophoresis for the quantification of biotinylated amplicons	69
Poly (dT) tailing for the species-specific probes	
Immobilization of the species-specific probes and controls onto the nylon membrane	70
Reverse hybridization, washing and detection	71
The specificity test of the species-specific probes	71
The sensitivity test of the species-specific probes	72
Healthy donors' sera screening for the <i>Candida albicans</i> using real-time PCR.	73
PCR amplification using ITS 1 and ITS 4 primers	73
Real-time PCR amplification using CAL 1 and CAL 3 primers	74
Double confirmation of the real-time PCR amplification	75
	76

	77
4 RESULTS	78
PCR amplification using ITS 3 and ITS 4 primers	78
Optimization of annealing temperature for species-specific primers via gradient PCR	79
Specificity of the species-specific primers in differentiating targeted <i>Candida</i> from other <i>Candida</i> ATCC control strains	
Specificity test for <i>Candida albicans</i> species-specific primers	84
Specificity test for <i>Candida dubliniensis</i> species-specific primers	
Specificity test for <i>Candida glabrata</i> species-specific primers	84
Specificity test for <i>Candida kefyr</i> species-specific primers	
Specificity test for <i>Candida krusei</i> species-specific primers	85
Specificity test for <i>Candida parapsilosis</i> species-specific primers	
Specificity test for <i>Candida rugosa</i> species-specific primers	86
Specificity test for <i>Candida tropicalis</i> species-specific primers	
The sensitivity of PCR detection method using <i>Candida albicans</i> species-specific primers	87
The sensitivity of nested-PCR detection method using <i>Candida albicans</i> species-specific primers	88
Optimization of DNA hybridization array for the detection of <i>Candida</i> species	89
Optimization of UV exposure time for immobilization of probes onto the membrane	90
Optimization of probe concentration in the DNA hybridization array	91
Reduction of hybridization time to 30 minutes	
Specificity of the species-specific probes in differentiating targeted <i>Candida</i> from other <i>Candida</i> ATCC control strains using DNA hybridization array	93
Specificity test for <i>Candida albicans</i> species-specific probe	
Specificity test for <i>Candida dubliniensis</i> species-specific probe	96
Specificity test for <i>Candida glabrata</i> species-specific probe	
Specificity test for <i>Candida kefyr</i> species-specific probe	
Specificity test for <i>Candida krusei</i> species-specific probe	96
Specificity test for <i>Candida parapsilosis</i> species-specific probe	
Specificity test for <i>Candida rugosa</i> species-specific probe	97
Specificity test for <i>Candida tropicalis</i> species-specific probe	
The differentiation of the mixture of <i>Candida</i> species DNA via DNA hybridization array	98
The sensitivity of the DNA hybridization array detection method using <i>Candida albicans</i> species-specific probe	
The screening of <i>Candida</i> clinical samples using species-specific primers via PCR	99

The screening of <i>Candida</i> clinical samples using species-specific probes via DNA hybridization array	99
The screening of healthy donor sera using CAL1/CAL3 primers via real-time PCR	101
	102
	103
	104
	105
	106
	107
	108
	109
	110
	112
	116
5 DISCUSSION	123
Overview	123
Nested-PCR using species-specific primers.	124
DNA hybridization array using species-specific probes.	
The limitations of the <i>Candida</i> identification methods in this study	137
Real-time PCR screening for healthy donor sera	148
	150
6 CONCLUSION	153
6.1 Future research recommendations	154
REFERENCES	156
APPENDICES	172
BIODATA OF THE AUTHOR	178
LIST OF PUBLICATIONS	179

LIST OF TABLES

Table		Page
2.1	The comparison of the duration, specificity, sensitivity, advantages and disadvantages of different <i>Candida</i> identification methods.	57
3.1	The species-specific primers length and their product sizes.	60
3.2	The species-specific probes G/C content and their length.	60
4.1	The range of annealing temperatures and optimum annealing temperatures for <i>Candida</i> species-specific primers, and their PCR product sizes.	92
4.2	The comparison between the efficacy and accuracy of different detection methods.	115
4.3	The summary of the result for the detection of <i>Candida albicans</i> in healthy donors' serum samples using CAL1 and CAL3 <i>Candida albicans</i> species-specific primers via real-time PCR.	117
5.1	The comparison of the estimated cost per reaction of different <i>Candida</i> species diagnostic methods with nested PCR and DNA hybridization array.	143

LIST OF FIGURES

Figure		Page
2.1	The schematic diagram of the nuclear ribosomal RNA internal transcribed spacer region.	31
2.2	The release of the fluorophore by 5' exonuclease activity of <i>Taq</i> polymerase in Taqman probe.	51
2.3	The structure of molecular beacons when it is unhybridized and hybridized to the target sequences.	52
2.4	The release of fluorescence signal from Scorpion primers.	53
4.1	Gel electrophoresis of ITS 3 and ITS 4 PCR products of <i>Candida</i> species.	78
4.2	Gel electrophoresis of <i>Candida albicans</i> and <i>Candida glabrata</i> species-specific primers gradient PCR products.	80
4.3	Gel electrophoresis of <i>Candida krusei</i> species-specific primers gradient PCR products.	80
4.4	Gel electrophoresis of <i>Candida dubliniensis</i> species-specific primers gradient PCR products.	81
4.5	Gel electrophoresis of <i>Candida kefyr</i> species-specific primers gradient PCR products.	81
4.6	Gel electrophoresis of <i>Candida rugosa</i> species-specific primers gradient PCR products.	82
4.7	Gel electrophoresis of <i>Candida parapsilosis</i> species-specific primers gradient PCR products.	82
4.8	Gel electrophoresis <i>Candida tropicalis</i> species-specific primers gradient PCR products.	83
4.9	Gel electrophoresis of PCR using <i>Candida albicans</i> species-specific primer with the extracted DNA of <i>Candida albicans</i> and 7 other <i>Candida</i> ATCC control strains.	84

4.10	Gel electrophoresis of PCR using <i>Candida dubliniensis</i> species-specific primer with the extracted DNA of <i>Candida dubliniensis</i> and 7 other <i>Candida</i> ATCC control strains.	85
4.11	Gel electrophoresis of PCR using <i>Candida glabrata</i> species-specific primer with the extracted DNA of <i>Candida glabrata</i> and 7 other <i>Candida</i> ATCC control strains.	86
4.12	Gel electrophoresis of PCR using <i>Candida kefyr</i> species-specific primer with the extracted DNA of <i>Candida kefyr</i> and 7 other <i>Candida</i> ATCC control strains.	87
4.13	Gel electrophoresis of PCR using <i>Candida krusei</i> species-specific primer with the extracted DNA of <i>Candida krusei</i> and 7 other <i>Candida</i> ATCC control strains.	88
4.14	Gel electrophoresis of PCR using <i>Candida parapsilosis</i> species-specific primer with the extracted DNA of <i>Candida parapsilosis</i> and 7 other <i>Candida</i> ATCC control strains.	89
4.15	Gel electrophoresis of PCR using <i>Candida rugosa</i> species-specific primer with the extracted DNA of <i>Candida rugosa</i> and 7 other <i>Candida</i> ATCC control strains.	90
4.16	Gel electrophoresis of PCR using <i>Candida tropicalis</i> species-specific primer with the extracted DNA of <i>Candida tropicalis</i> and 7 other <i>Candida</i> ATCC control strains.	91
4.17	Gel electrophoresis of PCR using <i>Candida albicans</i> species-specific primer with the extracted DNA of <i>Candida albicans</i> from the culture concentration of 1, 10, 10 ² , 10 ³ , 10 ⁴ , 10 ⁵ , 10 ⁶ , 10 ⁷ , and 10 ⁸ cell/mL.	93
4.18	Gel electrophoresis of PCR using <i>Candida albicans</i> species-specific primer using the 1 st round PCR products of <i>Candida albicans</i> from the simulated clinical serum sample as template with the concentration of 1, 10, 10 ² , 10 ³ , 10 ⁴ , 10 ⁵ , and 10 ⁶ cell/mL, respectively.	94
4.19	Membrane with different UV exposure for the immobilization of the species-specific probe onto it.	96

4.20	Membrane was labeled with different concentrations of probe and hybridized to the biotinylated PCR products in hybridization buffer.	97
4.21	Observation of the membrane when the duration of hybridization was reduced from 4 hours to 30 minutes.	98
4.22	DNA hybridization array template shows the specificity test of the <i>Candida albicans</i> species-specific probe.	99
4.23	DNA hybridization array template shows the specificity test of the <i>Candida dubliniensis</i> species.	101
4.24	DNA hybridization array template shows the specificity test of the <i>Candida glabrata</i> species.	102
4.25	DNA hybridization array template shows the specificity test of the <i>Candida kefyr</i> species.	103
4.26	DNA hybridization array template shows the specificity test of the <i>Candida krusei</i> species.	104
4.27	DNA hybridization array template shows the specificity test of the <i>Candida parapsilosis</i> species.	105
4.28	DNA hybridization array template shows the specificity test of the <i>Candida rugosa</i> species.	106
4.29	DNA hybridization array template shows the specificity test of the <i>Candida tropicalis</i> species.	107
4.30	DNA hybridization array template shows the differentiation of the mixture of <i>Candida</i> species DNA using <i>Candida</i> species-specific probes.	108
4.31	DNA hybridization array using <i>Candida albicans</i> species-specific probe with the biotin-labeled amplicon of <i>Candida albicans</i> from the culture concentration of 1, 10, 10 ² , 10 ³ , 10 ⁴ , 10 ⁵ , 10 ⁶ , 10 ⁷ , and 10 ⁸ cell/mL.	109

4.32	Gel electrophoresis of PCR using <i>Candida albicans</i> , <i>Candida dubliniensis</i> , <i>Candida glabrata</i> , <i>Candida krusei</i> , <i>Candida parapsilosis</i> , <i>Candida rugosa</i> , and <i>Candida tropicalis</i> species-specific primers with the extracted DNA of <i>Candida</i> clinical samples.	111
4.33a	DNA hybridization array template shows the result of the screening of <i>Candida</i> clinical samples using species-specific probes.	113
4.33b	DNA hybridization array template shows the result of the screening of <i>Candida</i> clinical samples using species-specific probes.	114
4.34	Melting curve analysis of serum samples screening and gel electrophoresis of real-time PCR products (Part 1).	118
4.35	Melting curve analysis of serum samples screening and gel electrophoresis of real-time PCR products (Part 2).	119
4.36	Melting curve analysis of serum samples screening and gel electrophoresis of real-time PCR products (Part 3).	120
4.37	Melting curve analysis of serum samples screening and gel electrophoresis of real-time PCR products (Part 4).	121
4.38	Melting curve analysis of serum samples screening and gel electrophoresis of real-time PCR products (Part 5).	122

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bps	Base pairs
CFU	Colony forming units
dNTP	Deoxynucleotide triphosphate
EDTA	Disodium ethylene-diaminetetra acetate.2H ₂ O
EIA or ELISA	Enzyme-linked Immunosorbent assay
EtBr	Ethidium bromide
ITS	Internal Transcribed Spacer
KCl	Potassium chloride
KH ₂ PO ₄	Potassium dihydrogen phosphate
LiPA	Line probe assay
MgCl ₂	Magnesium chloride
MW	Molecular weight
Na ₂ HPO ₄	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitro-blue-tetrazolium
NH ₄ SO ₄	Ammonium sulfate
n-PCR	Nested-PCR