LAPORAN AKHIR GERAN PENYELIDIKAN
USM JANGKA PENDEK

TAJUK:

Development of Rapid Screening Method for the Diagnostic of Fragile X Syndrome Using Real Time PCR.

304/PPSP/6131490

15 Mac 2007 – 14 Mac 2009

Dr. Hoh Boon Peng

SENARAI SEMAKAN UNTUK BUKU LAPORAN AKHIR GERAN USM JANGKA PENDEK

NAMA PENYELIDIK UTAMA	:	**Dr. Hoh Boon Peng**
NAMA CO-RESEARCHER	:	**Prof. Madya Dr. Zilfalil Bin Alwi**
TAJUK GERAN	:	**Development of Rapid Screening Method for the Diagnostic of Fragile-X Syndrome Using Real Time PCR.**
NO.AKAUN	:	**304/PPSP/6131490**

SENARAI SEMAKAN SEMASA PENYERAHAN BUKU LAPORAN AKHIR
(Sila Tandakan (4) Pada Kotak Yang Berkenaan)

NO.	PERKARA	ADA	TIADA
1.	Borang Laporan Akhir Projek Penyelidikan USM Jangka Pendek	✓	
2.	Borang Laporan Hasil Penyelidikan, PPSP	✓	
3.	i) Salinan Menuskrip		
	ii) Salinan surat/email bukti penghantaran kepada mana-mana journal		
4.	Penyata Perbelanjaan (Financial Statement) (Sila dapatkan daripada Jabatan Bendahari)	✓	
5.	Laporan Komprehensif (termasuk kertas persidangan atau seminar dan penerbitan saintifik hasil daripada projek ini)	✓	
6.	Surat pemakluman penghantaran Laporan Akhir ke Bhg. Penyelidikan	✓	

Nota:

* Sila buat 3 salinan buku laporan Akhir
* No. 1-5 - Perlu dimasukkan dalam Buku Laporan Akhir
* No.6 - Hantar terus Kepada Pn. Che Merah Ismail (RCMO) hanya salinan kepada Bhg. R&D, PPSP

My doc/checklist borang2/sue

UNIVERSITI SAINS MALAYSIA

LAPORAN AKHIR PROJEK PENYELIDIKAN JANGKA *PENDEK*

FINAL REPORT OF SHORT TERM RESEARCH PROJECT

Sila kemukakan laporan akhir ini melalui Jawatankuasa Penyelidikan di Pusat Pengajian dan Dekan/Pengarah/Ketua Jabatan kepada Pejabat Pelantar Penyelidikan

1. **Nama Ketua Penyelidik:** *Name of Research Leader* **Hoh Boon Peng**

☐ Professor Madya/ *Assoc. Prof* ☑ Dr./ *Dr.* ☐ Encik/Puan/Cik *Mr/Mrs/Ms*

TERIMA
21 DEC 2008
PEJABAT DEKAN
PELANTAR PENYELIDIKAN KLINIKAL
UNIVERSITI SAINS MALAYSIA
KAMPUS KESIHATAN

2. **Pusat Tanggungjawab (PTJ):**
School/Department

Pusat Pengajian Sains Perubatan

3. **Nama Penyelidik Bersama:**
Name of Co-Researcher *Prof. Madya Dr. Zulfaki Alwi*

4. **Tajuk Projek:**
Title of Project *Development of Rapid Screening Method for the Diagnostic of Fragile-X Syndrome Using Real Time PCR*

5. **Ringkasan Penilaian/***Summary of Assessment*	**Tidak Memuaskan** *Inadequate* 1 2	**Boleh Diterima** *Acceptable* 3	**Sangat Baik** *Very Good* 4 5
i) **Pencapaian objektif projek:** *Achievement of project objectives*	☐ ☐	☐	☑ ☐
ii) **Kualiti output:** *Quality of outputs*	☐ ☐	☑	☐ ☐
iii) **Kualiti impak:** *Quality of impacts*	☐ ☐	☐	☑ ☐
iv) **Pemindahan teknologi/potensi pengkomersialan:** *Technology transfer/commercialization potential*	☐ ☐	☐	☑ ☐
v) **Kualiti dan usahasama :** *Quality and intensity of collaboration*	☐ ☐	☐	☑ ☐
vi) **Penilaian kepentingan secara keseluruhan:** *Overall assessment of benefits*	☐ ☐	☐	☑ ☐

6. Abstrak Penyelidikan
(Perlu disediakan di antara 100 - 200 perkataan di dalam Bahasa Malaysia dan juga Bahasa Inggeris. Abstrak ini akan dimuatkan dalam Laporan Tahunan Bahagian Penyelidikan & Inovasi sebagai satu cara untuk menyampaikan dapatan projek masing-masing kepada pihak Universiti & masyarakat luar).

Abstract of Research
(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English).
This abstract will be included in the Annual Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researchers to the University and the community at large)

Fragile X Syndrome (FXS) is the most prevalent inherited cause of mental retardation. It is caused by the CGG repeat instability in FMR1 gene, located on Xq27.3. In normal individuals, the CGG repeats range from 5 to 53. In pre-mutation carriers, 60 to 200 and more than 200 repeats in full mutation patients. FXS patients have variable clinical features and because of that, an accurate clinical diagnosis is always a problem. Currently, Cytogenetic, PCR and Southern Blot are widely used for diagnosis of FXS. The present method of FXS diagnosis is complicated and labor intensive. So, Real Time PCR technique was developed for an easy, rapid and reliable diagnosis. TaqMan chemistry was used in this PCR. Standard curve were developed from 4 controlled DNA. The CGG repeats number was calculated from the standard curve by using the Light Cycler Software 4.05. All the normal and permutation samples were successfully amplified and calculated. The amplification of full mutation samples was successful. However the slippage during PCR amplification occasionally happened resulting in inconsistency output. So, futher study on the full mutation amplification must be done.

Remark: This study was expected to be completed by March 2009. However, it is ended earlier than expected due to my resignation as an academic staff in School of Medical Sciences, USM, effective from January 2009.

7. Sila sediakan laporan teknikal lengkap yang menerangkan keseluruhan projek ini.
[Sila gunakan kertas berasingan]
Applicant are required to prepare a Comprehensive Technical Report explaning the project.
(This report must be appended separately)

Refer attachment

Senaraikan kata kunci yang mencerminkan penyelidikan anda:
List the key words that reflects your research:

Bahasa Malaysia	Bahasa Inggeris
Pramutasi	Premutation
Mutasi lengkap	Full Mutation
Graf piawai	Standard curve

8. Output dan Faedah Projek
Output and Benefits of Project

(a) * **Penerbitan Jurnal**
Publication of Journals
(Sila nyatakan jenis, tajuk, pengarang/editor, tahun terbitan dan di mana telah diterbit/diserahkan)
(State type, title, author/editor, publication year and where it has been published/submitted)

Refer attachment

(b) **Faedah-faedah lain seperti perkembangan produk, pengkomersialan produk/pendaftaran paten atau impak kepada dasar dan masyarakat.**
State other benefits such as product development, product commercialisation/patent registration or impact on source and society.

The study has shed light in general, the PCR amplification of the DNA fragments with highly GC content, and inclusion of additive components which had made successful amplification in the Real-Time PCR technology. Specifically, the success in amplifying the FMR1 CGG repeat of various stages provides a stepping stone towards the development of an alternative diagnosis of Fragile X syndrome. Though inconsistency of the full mutated stage amplification was observed, the hurdle can be overcome with a little further optimization.

* Sila berikan salinan/*Kindly provide copies*

(c) **Latihan Sumber Manusia**
Training in Human Resources

i) Pelajar Sarjana: One Master Student
Graduate Students
(Perincikan nama, ijazah dan status)
(Provide names, degrees and status)

Maryana Hikmah Binti Elias : Master of Science (Human Genetics)

Status : Second Semester

ii) Lain-lain:
Others

9. **Peralatan yang Telah Dibeli:**
Equipment that has been purchased

Tandatangan Penyelidik
Signature of Researcher

HOH BOON PENG, PhD
LECTURER
HUMAN GENOME CENTRE
SCHOOL OF MEDICAL SCIENCE HEALTH CAMPUS,
UNIVERSITI SAINS MALAYSIA, KUBANG KERIAN.

21/2/08

Tarikh
Date

Komen Jawatankuasa Penyelidikan Pusat Pengajian/Pusat
Comments by the Research Committees of Schools/Centres

Laporan akhir ini telah dinilai oleh seorang penilai luar serta disemakan oleh JK penyelidikan PPP sebelum dihantar lengkap

Antara "output" dari projek penyelidikan ini adalah beberapa penerbitan serta cetak salinan dari "journal submission" ke International Medical Journal bertarikh 21 April 2009

PROFESSOR AHMAD SUKARI HALIM
Chairman of Research Commitee
School of Medical Sciences
Health Campus
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan

TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENGAJIAN/PUSAT
Signature of Chairman
[Research Committee of School/Centre]

4/5/09
Tarikh
Date

amplified and calculated. The amplification of full mutation samples was successful. However the slippage during PCR amplification occasionally happened resulting in inconsistency output. So, further study on the full mutation amplification must be done.

Remark: This study was expected to be completed by March 2009. However, it is ended earlier than expected due to my resignation as an academic staff in School of Medical Sciences, USM, effective from January 2009.

Comprehensive Technical Report

Real-time PCR was done to monitor the fluorescence emitted during the reaction as an indicator of the amplicon production during each PCR cycle in real time as opposed to the endpoint detection. The Real Time PCR experimental protocol was being setup in the Light Cycler® 1.5 Instrument (Roche Applied Science) using the Light Cycler® Software Version 4.05. The protocol of the amplification consisted of three programs. The programs are the hot start followed by the amplification and cooling procedure.

After a series of optimization using the conventional PCR with SYBR Green, the primer dimer was still a major problem. To overcome this, TaqMan technology was applied. The reagents used for the Real-time PCR amplification was the Light Cycler® TaqMan® Master kit supplied by Roche. The probe was designed using the Universal Probe Library Software (www.universalprobelibrary.com). The probes sequence designed were added with new technology which is the duplex-stabilizing DNA analogue LNA (Locked Nucleic Acid) and was much shorter than the classic TaqMan probe, which is 25 to 35 nucleotide probes. However, it had a high Melting temperature (Tm) yet more stable. The probe designed was the probe #31 (cat. no. 04687647001) with sequence 5'-TGG TGG AA-3'. All the primers were designed by using Primer3 Software, version 0.4.0 (http://fokker.wi.mit.edu/primer3/input.htm). Forward primer was 5'-CTC CGT TTC GGT TTC ACT TC-3' and reverse primer was 5'-GTA CCT TGT AGA AAG CGC CAT T-3'.

To confirm if the primers designed were correct and the sequences amplified are the sequence of interest located at the 5' Untranslated Region of the FMR1 gene, some of the amplified samples were sequenced. The results obtained from the normal controlled samples with 30 CGG repeat showed the specificity of the primers. However, samples with more than 60 CGG repeats were failed to amplify due to the high GC content.

Like conventional PCR, high GC content leads to difficulty or even inhibit the amplification process. The difficulties of amplifying the high GC content sequences are mostly due to the propensity of the repeats to form stable secondary structure in vitro. To overcome this major problem, some additives were added namely: Betaine, 7-deaza-dGTP, DMSO and formamide. Serial optimizations of the additives concentration were done in order to obtain the best amplification and was found also that 7-deaza-dGTP do not lead to inhibition of the probe's fluorescent detection on the Real Time PCR as it does in Ethidium Bromide.

Standard curve were developed from 7 DNA controls. These DNA controls which had been analyzed by Southern Blot were obtained from University of Amsterdam via collaboration between the Universities. The samples were normal repeats (30 CGG repeats and 45 CGG repeats), permutation (60 CGG repeats and 100-200 CGG repeats), full mutation with more than 200 CGG repeats, more than 300 CGG repeats and more than 500 CGG repeats. The CGG repeats number of the samples was calculated from the standard curve by using the Light Cycler Software 4.05. All the normal and permutation samples were successfully amplified and calculated.

Even though the amplification of full mutation samples was successful, inconsistency of results occasionally happened, probably due to the slippage during PCR amplification. Following the hairpins structure, longer CGG repeat tracts have the possibility to form slipped strand DNA in the middle of amplification. So, further study of the slippage on the full mutation amplification and the PCR stabilization should be carried out in the near future.

Presentation in Conferences:

1. Medical Genetic Conference 2007; Poster presentation on "Is Fragile X Syndrome Underdiagnose through Cytogenetic Analysis Alone". 16th - 19th November 2007

2. 13th National Conference of Medical Sciences; Oral presentation on "Fragile X Syndrome is Under Diagnose through Cytogenetic Analysis Alone: 6 Years of HUSM Experience. 22nd - 23rd May 2008.

3. 10th Symposium of the Malaysian Society of Applied Biology; Poster presentation on "Fragile X Syndrome among Siblings - Difference in Cytogenetic and Molecular Genetic Analysis". 6 - 8 November 2008.

Manuscript under preparation & to be submitted:

1. Fragile X Syndrome among Siblings – Difference in Cytogenetic and Molecular Genetic Analysis Results.

2. Addition Of Low-Cost Additives Improves Real Time PCR Assay of GC Rich FMR1 Region Using TaqMan Probe.

BORANG LAPORAN HASIL PENYELIDIKAN
PPSP

Tajuk geran: **Development of Rapid Screening Method for the Diagnostic of Fragile X Syndrome Using Real Time PCR.**
Penyelidik: **Dr. Hoh Boon Peng**
Jenis geran: **Short Term Grant**
Tempoh geran: **15 Mac 2007 – 14 Mac 2009**

Jenis laporan: Laporan Kemajuan [ ] Alatan di beli [ ] Ya:nyatakan.....
Laporan Akhir*: [✓] [✓] Tidak

OBJEKTIF SPESIFIK KAJIAN (sama spt dalam proposal asal)	**SECARA RINGKAS TERANGKAN PENCAPAIAN/HASIL**	**OBJEKTIF TERCAPAI ATAU TIDAK**
1. To develop and optimize a rapid, easy and reliable method for the diagnosis of Fragile-X Syndrome, compared to the existing technique.	Real Time PCR technique was developed for an easy, rapid and reliable diagnosis. TaqMan chemistry was used in this PCR. Standard curve were developed from 4 controlled DNA. Amplification was successful for all normal, pre-mutated and full mutated DNA. However, inconsistency were shown due to the long fragment of PCR and high GC content in full mutated stage (>96%).	Tercapai
2.		
3.		
4.		

- *Laporan Akhir perlu disertakan salinan manuskrip dan surat yang dihantar kepada mana-mana jurnal untuk penerbitan.*

Nama Penyelidik Utama (PI): **Dr. Hoh Boon Peng** t.t.:
Tarikh: **22 December 2008**

T. Sakuta M. D.,
IMJ Division,
Seronjihasha C0., Ltd.
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Setagaya-ku,
Tokyo, 154-0005, Japan

21 April 2009

Dear Editor

It gives us great pleasure to submit a paper to the International Medical Journal. The title of the manuscript is: **Divergence in Cytogenetic and Molecular Genetic Analysis Results – A Case Report of Fragile X Syndrome among Siblings.** Here we report a pair of brothers suspected to be FXS patients with similar clinical features. We used the cytogenetic analysis along with the molecular analysis; fragment analysis, methylation specific PCR and Southern blot to diagnose the Fragile X Syndrome. However, the cytogenetic result for younger brother did not show fragile site at Xq27.3 of the X chromosome while molecular result was confirmatory for FXS. Conversely, the elder brother showed confirmatory results for Fragile X mutation in both cytogenetic and molecular analysis. This results show an interesting finding in the divergence of both the technique.

We hereby declare that this is an original work.

We appreciate you for considering this manuscript for publication.

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DIVERGENCE IN CYTOGENETIC AND MOLECULAR GENETIC ANALYSIS RESULTS – A CASE REPORT OF FRAGILE X SYNDROME AMONG SIBLINGS.

[1]Elias Marjanu Hikmah, [1]Mamat Norhashimah , [1]Ismail Siti Mariam, [2]Salmi Razak, [3]Pornprot Limprasert, [3]Wanna Sudhikaran, [1]Ravindran Ankathil, [1,2]Bin Alwi Zilfalil.

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ABSTRACT

Fragile X Syndrome (FXS) is the most prevalent inherited cause of mental retardation. The prevalence of FXS in males and females are approximately 1 in 4000 and 1 in 8000 respectively. It is caused by CGG repeat instability in the FMR1 gene, located on chromosome Xq27.3. Normal individuals have CGG repeats ranging from 5 to 53. In premutation carriers, the CGG repeats range from 60 to 200 and shall be more than 200 repeats for full mutation patients. FXS patients have variable clinical features and because of that, an accurate clinical diagnosis is always a problem. Currently, Cytogenetic, PCR and Southern Blot Techniques are widely used for diagnosis of FXS. Here we report a pair of brothers suspected to be FXS patients with similar clinical features. However, the cytogenetic result for younger brother did not show fragile site at Xq27.3 of the X chromosome while molecular result was confirmatory for FXS. Conversely, the elder brother showed confirmatory results for Fragile X mutation in both cytogenetic and molecular analysis. We therefore conclude that cytogenetic analysis alone cannot be dependable for the confirmatory diagnosis of FXS.

KEY WORDS

Fragile X Syndrome, cytogenetic analysis, Southern Blot, FMR1 gene and CGG repeats.

Design: A case-control study.

INTRODUCTION

The Fragile X Syndrome (FXS), OMIM#300624, is the most common form of inherited mental retardation. The prevalence of Fragile X syndrome in males and females are approximately 1 in 4000 and 1 in 8000 respectively (Crawford *et al.*, 2001). Approximately 1 in 700 females are carriers and the frequency of premutation in general population is approximately 1 in 259 females and 1 in 813 males (Rousseau *et al.*, 1995; .Turner *et al.*, 1996). However, recent population studies have indicated a much higher prevalence of the Fragile X Syndrome. Hagerman (2008) reported that the best current estimate for the frequency of the FM in females is 1 in 2500 and 1 in 3600 males.

This syndrome was the first genetic disease identified that is caused by CGG repeat instability. This unstable repeat is in the FMR1 gene, located on the Xq27.3 (Verkerk et al., 1991) of the X chromosome. FMR1 gene consists of 17 exons and is roughly 38 kb in size. Normally, the CGG repeats are stable with a range of 5 to 53 repeats. In premutation, the CGG repeats are between 60 and 200 and the instability is emphasized as the number of repeats increases. When 200 or more CGG repeats are present, FMR1 gene is usually hypermethylated, which is associated with transcriptional silencing of the gene that is commonly referred to as the FMR1 full mutation (FM) (Reiss *et al.*, 1995). The FM prevents transcription of the FMR1-gene, resulting in the absence