香港醫學遺傳學會
Hong Kong Society of Medical Genetics

HKSMG Newsletter

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Message from the Editor

It is time again for another issue of our Newsletter. The last issue featured a short article on medical practice – *Genetic Study of CNS Diseases*. In this issue, we feature a short article on technology update – *Frequently Asked Questions about HRM Analysis*. We hope to update the members, scientists and clinicians alike, on the new technology HRM analysis in a concise and informal fashion. Do not know what this abbreviation HRM stands for! Then, you have to read this Newsletter.

To encourage sharing of views and experience, you are most welcome to submit short articles for future issues of our Newsletter. You are also welcome to pass this Newsletter to your colleagues and friends. At the same time, you can also encourage them to join our Society.

Please be informed that our 10th Annual General Meeting and the accompanying mini-symposium are scheduled on 30 May 2009 (Saturday). The theme of the mini-symposium is *Genetics of Neurodegenerative Diseases*. Please mark your diary. See you in the coming Annual General Meeting and mini-symposium.

Yip Shea Ping
Editor

Reminder of Annual Subscription

Please be reminded to pay your overdue subscription as soon as possible. All new or re-joining subscribers will be waived for the joining fee of HKD200 before 30 May 2009 for the year 2009/2010. In the past, society members who had discontinued their subscriptions would have to pay the joining fee again. As the Society is now planning a whole series of scientific seminars in the coming months and the 9th Asia-Pacific Conference on Human Genetics in 2010, there might be exclusive exemptions or discounts on the registration fees for society members. If you did not subscribe your membership in 2008/2009 or in the years before, it would be a good time to save by re-activating your membership. Please refer to the end of this newsletter for the 2009/2010 Membership Renewal Form.

Priscilla Poon
Hon. Secretary

Report on scientific activity in February 2009

A scientific seminar entitled *Marker Chromosomes* was held on 4 February 2009 at the Federation of Medical Societies of Hong Kong. It was a seminar jointly organized by our Society, the Hong Kong Society of Cytogenetics and the Hong Kong Branch of the Institute of Biomedical Science. There were about 50 participants attending the seminar.

The speaker was Professor Konstantin Miller. Prof. Miller is the Head of the Clinical Cytogenetic Laboratories at the Institute of Human Genetics, Hannover Medical University, Germany. He is very active in the field of cytogenetics and has been involved in organising external quality assessment programmes in cytogenetics since 1993. Prof. Miller is also very active academically and has published over 60 scientific articles and presented extensively in international meetings. In the seminar, he shared with the audience his experience in studying marker chromosomes. By definition, a marker chromosome (mar) is a structurally abnormal chromosome that cannot be unambiguously identified or characterized by conventional banding technique. In his experience, most marker chromosomes were bisatellited and derived from chromosomes 15 or 22, and multi-colour fluorescence *in situ* hybridization (MFISH) was a useful tool in characterizing a marker chromosome.

Frequently asked questions about HRM analysis

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Introduction

The technology of HRM analysis has been around for some years. More and more instruments have features capability of HRM analysis. It has great potential in molecular diagnostics and is also increasingly used in research setting. In this short article, this technology is purposely introduced in the form of questions and answers to make it both interesting and comprehensible to both scientists and clinicians.

Q. What is HRM?

A. If you search "HRM" in the internet browser Google, over 9 million hits return with an overwhelming majority of the hits telling you that HRM represents "human resource management". If you key in "mutation" in the "Search within results" box, you get more than 13,000 hits containing "HRM" and "mutation". The term "HRM" of interest to us refers to high resolution melting. If you then search "high resolution melting" in PubMed, you get over 700 articles related to this topic.

HRM analysis is a new polymerase chain reaction (PCR)-based method that can be used to genotype known sequence variations and/or screen unknown sequence variations in an amplicon in the presence of a special type of double-stranded (ds) DNA binding dye. The sequence variations can be either polymorphisms or disease-causing mutations. This method was first reported by a group led by Carl Wittwer in 2003.[1] In other words, this technology has a history of about 6 years only.

Q. Given that there are many different methods of genotyping and mutation screening in common use, what is so special about HRM analysis?

A. In the simplest case, you only need standard PCR reaction components and a special dsDNA binding dye. Once standard PCR cycles are completed, HRM analysis can be finished in about 10 minutes. As such, HRM analysis is a rapid, low-cost PCR-based method for genotyping and screening sequence variations with very little manual manipulations.

Q. What are required for performing HRM analysis?

A. In addition to the components of a standard PCR, three key components are required for performing HRM analysis: (1) a saturating dye, (2) a special high-precision instrument, and (3) a software package that features the required algorithms for data analysis.

Q. What is a saturating dye?

A. Ethidium bromide is required for visualizing DNA fragments in agarose gel, but is not sensitive enough for continuous monitoring of real time PCR and classical melting curve analysis. SYBR Green I is used for monitoring real time PCR and classical melting curve analysis, but is not good enough for HRM analysis. If SYBR Green I is used at concentrations high enough to saturate all binding sites of dsDNA, it will inhibit PCR. With increasing temperature, a low-melting domain of dsDNA melts first and the dye molecules at a non-saturating concentration re-distribute or jump from the low-melting domain to other high melting domains without changing the fluorescence signal. Therefore, the fluorescence intensity does not reflect accurately minor alterations in melting temperature in the presence of a non-saturating dye. As such, non-saturating dyes are not good enough for HRM analysis.

A saturating dye is a dsDNA binding dye that can be used at concentrations high enough to saturate all binding sites of dsDNA without inhibiting PCR too much. With increasing temperature, a low-melting domain of dsDNA melts first and the dye molecules at a saturating concentration are released without re-distribution to other high melting domains of dsDNA. This results in a reduction of the fluorescence intensity. Therefore, a saturating dye can be used to monitor minor changes in melting temperatures. A saturating dye is required for HRM analysis. Saturating dyes in common use include LCGreen Plus (Idaho Technology), ResoLight (Roche), Syto 9 (Invitrogen), EvaGreen (Biotum) and others.

Q. What features are required for high-precision instruments dedicated for HRM analysis?

A. High-precision instruments for HRM analysis must be able to maintain highly uniform temperatures across all reaction wells even during ramping and acquire enough data points per °C.

HRM analysis serves to discriminate minute differences in melting curves. The larger the amplicons are, the smaller the differences become. This makes the precision of the instruments for HRM analysis even more critical. Such high-precision instruments come in two formats: (1) real time thermal cyclers with HRM capability, e.g. LightCycler 480 (Roche), and Rotor-Gene Q (Qiagen; the same as Rotor-Gene 6000 from Corbett Research, which was recently acquired by Qiagen); and (2) stand-alone instruments dedicated for HRM analysis, e.g. HR-1 and LightScanner (both from Idaho Technology). PCR products must be transferred to reaction tubes/wells designated for use with stand-alone instruments before HRM analysis.

These instruments vary in the number of reaction wells per run, the turnaround time, the format of reaction tubes/wells, economy and PCR integration. Comparison of instruments for HRM analysis has been published recently.[2-4]

Q. What are the essential features of a software package for HRM analysis?

A. The steps involved in the analysis of melting data by HRM software include: (1) normalization of fluorescence intensity, (2) temperature-shifting, and (3) presentation of HRM data in the form of difference plots. Software packages for HRM analysis must have these three basic features required for handling HRM data. For HRM analysis, it is essential to have specific PCR. This means only a single PCR product is obtained. Otherwise, analysis of HRM data may not yield meaningful results.

Q. What is fluorescence normalization?

A. When amplicons are melted, fluorescence intensity is high at low temperatures and becomes low with increasing temperatures. A plot of fluorescence intensity (Y axis) is plotted against the temperature (X axis) (**Fig 1A**). The pre-melting maximum fluorescence intensity usually varies from sample to sample. For each individual sample, the maximum fluorescence intensity is set as 100% and the intensities at other temperatures are expressed as a percentage of the pre-melting maximum intensity. Thus, normalization against fluorescence (Y axis) plots all melting curves between 100% (completely double-stranded) and 0% (completely single-stranded) fluorescence intensity (**Fig 1B**). Normalization of fluorescence intensity makes comparison of melting transitions much easier.

Q. What is temperature shifting?

A. There bounds to be some variation in temperature across the samples in the sample block and all samples are not identical because of variation in pipetting and evaporation. These differences can be minimized by shifting the temperature axis (X axis) of the fluorescence-normalized melting curves to a point where the dsDNA is completely denatured (**Fig 1C**). This is known as temperature shifting (also known as curve overlay), which makes the identification of heteroduplexes and hence heterozygous genotypes much easier and more distinctive. However, the homozygous variants become more difficult to be identified. Therefore, it is worth examining the HRM data with and without temperature shifting.

Q. What is a difference plot?

A. Negative first derivative plots (-dF/dT) have been used extensively to show the melting peaks and hence the melting temperatures (Tm) for labeled probes and amplicons. Two melting peaks can easily be distinguished from each other if the Tm difference is at least 4 – 5°C. However, this is not the case when the Tm difference is less than 2°C. The small differences in the original normalized temperature-shifted melting curves can be magnified by plotting the difference between a reference curve (usually the homozygous wild-type genotype) and all other melting curves (**Fig. 1D**). This is the difference plot. The distinctive shapes of the difference plots corresponding to the different genotypes make it easy for visual discrimination. Statistical clustering algorithms can also be used to automatically classify the difference plots into different groups corresponding to different genotypes.

Q. What are the applications of HRM analysis?

A. First, HRM analysis can be used to genotype known sequence variations in small amplicons. This eliminates the use of labeled probes. This is good enough for genotyping sequence variations with the following allelic pairs: C/T, G/A, C/A and G/T, which account for more than 84% of human single nucleotide polymorphisms. Genotyping of A/T or G/C allelic pairs is less reliable with small amplicon melting because the Tm difference is generally less than 0.4°C and cannot be distinguished reliably

even by difference plots.

Second, HRM analysis can determine quickly if the corresponding sequences (e.g. HLA sequences) from two or more individuals are the same or not by melting the samples individually and in mixture. The interest here is the matching of the sequence rather the actual DNA sequence and hence no DNA sequencing is required.

Third, HRM analysis can be used to detect rare sequence variants in a high background of wild-type sequences (e.g. rare tumor cells with mutation in the presence of many normal cells without the mutation). For detection, HRM analysis requires mutant DNA to be present at 2% or more while DNA sequencing requires mutant DNA to be present at 20% or more. In other words, HRM analysis is more sensitive. In addition, HRM analysis is also found to increase the sensitivity and precision of methylation analysis although it is not essential for such analysis. Note that methylation of cytosine to 5-methylcytosine usually occurs within CpG islands near the gene promoters and is important in regulating the expression levels of genes concerned.

Last but not least, HRM analysis can be used to screen unknown mutations. It can screen out normal samples quickly and produce variant difference plots for samples with mutations, which can then be characterized by DNA sequencing. In other words, the number of samples that have to be sequenced can be greatly reduced by the initial HRM analysis. The sensitivity and specificity of HRM analysis to detect heterozygous single base changes were found to be 100% for amplicons less than 400 basepairs.[5] The use of HRM analysis for mutation scanning has also been evaluated by a national genetics reference laboratory and the report can be downloaded free of charge.[6]

References

1. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 2003; **49**: 853-60.
2. Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. *Clin Chem* 2006; **52**: 494-503.
3. Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. Instrument comparison for heterozygote scanning of single and double heterozygotes: a correction and extension of Herrmann et al., Clin Chem 2006;52:494-503. *Clin Chem* 2007; **53**: 150-2.
4. Herrmann MG, Durtschi JD, Wittwer CT, Voelkerding KV. Expanded instrument comparison of amplicon DNA melting analysis for mutation scanning and genotyping. *Clin Chem* 2007; **53**: 1544-8.
5. Reed GH, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin Chem* 2004; **50**: 1748-54.
6. National Genetics Reference Laboratory (Wessex). Mutation scanning by high resolution melt analysis. Evaluation of Rotor-Gene™ 6000 (Corbett Life Science), HR-1™ and 384 well LightScanner™ (Idaho Technology). June 2006. (http://www.ngrl.org.uk/Wessex/downloads)

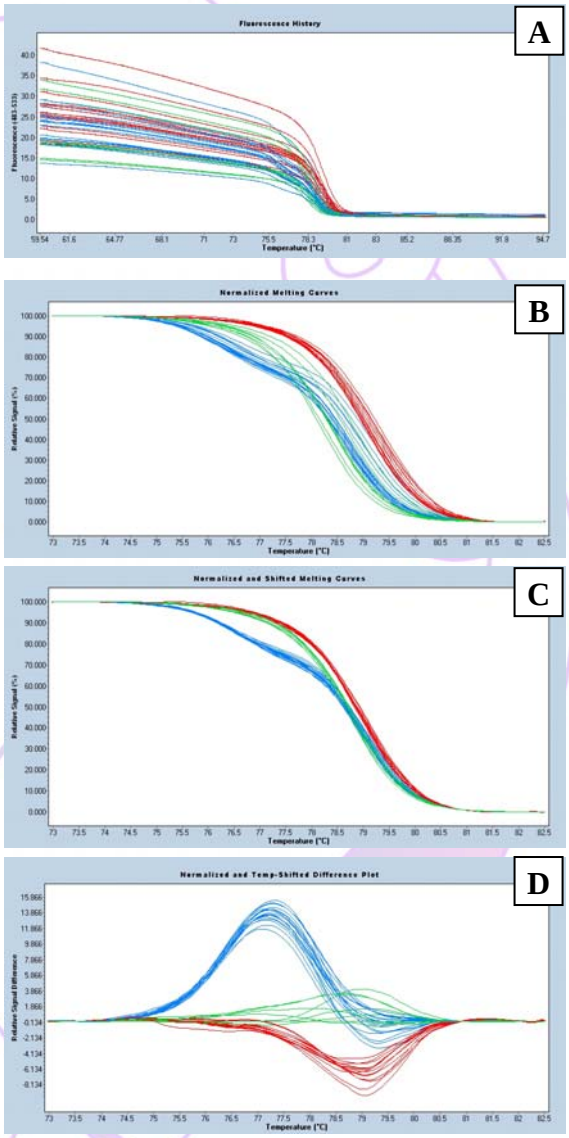

Fig. 1. Analysis of HRM data. In HRM analysis, the fluorescence intensity is first plotted against temperature (A). The fluorescence intensity is then normalized (B) and the temperature axis shifted to the point where the dsDNA is completed denatured (C). Finally, the differences between a reference curve and all other melting curves are plotted as difference plots (D). The three different colours of the curves represent the three different genotypes of a biallelic single nucleotide polymorphism carried in an amplicon of 56bp.

HONG KONG SOCIETY OF MEDICAL GENETICS

10TH AGM & MINI-SYMPOSIUM

"Genetics of Neurodegenerative Diseases"

Program:

17:00-17:30	**Registration**
17:30-18:15	**"Introduction to genetics of brain diseases"** *Prof Larry Baum, School of Pharmacy, The Chinese University of Hong Kong*
18:15-19:00	**"Genome-wide association studies and genetic risk prediction in dementia"** *Prof Pak Sham, Head of the Department of Psychiatry, The University of Hong Kong*
19:00-19:30	**Annual General Meeting**

Date: 30 May 2009 (Saturday)

Time: 17:00-19:30

Venue: The Federation of Medical Societies of Hong Kong
Duke of Windsor Social Service Building
Lecture Hall, 4/F, 15 Hennessy Road, Wanchai, Hong Kong

Annual Dinner

All subscribed HKSMG members are cordially invited to the annual dinner after the AGM. Reservation is on a first-come-first serve basis. Please renew your HKSMG Membership before 30 May 2009.

Reservation and Enquiries:

Dr. Priscilla Poon at E-mail: hksmg.com@gmail.com

CME & CPD applications in progress

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Membership Renewal Form for 2009/2010

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Membership Category	Subscription Fees	Amount Paid (HK$)
Ordinary	$200	
Associate	$150	
Associate (Overseas)	$100	

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c/o Mr Chan Wing Kwong
Department of Obstetrics and Gynaecology
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