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# An Automatic Fast High Resolution HDL Subfractioning Assay Using Microfluidic Electrophoretic System

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## Abstract

***Objectives:*** *Studies in recent decade demonstrated the importance of HDL subfractionation in precision medicine. It is notable that not all HDL subfractions are "good" lipoprotein. The lack of a reliable technique that can precisely identify each particle of HDL subfractions remains the major obstacle in clinical application. Based on the principle of microfluidic electrophoresis, we have developed an automatic system (MFE) that meets the need of clinical applications. The objectives of this paper is to test the system for its application, precision, and also the normal range in healthy population using one of major subfraction, HDL-2b, as a target.*

***Methods:*** *The MFE was compared with traditional non-denaturing polyacrylamide gradient gel electrophoresis (GGE). Intra-chip, inter-chip, inter-instrument, inter-lab, and inter-operator precisions were all tested by using the sample in various conditions. An instruction was provided with the instrument and HDL subfractioning kit. Data were analyzed with SPSS for correlation of variation, regressions, etc.*

***Results:*** *The new system exhibited a linear correlation with the traditional GGE system. The intra-chip %CVs, inter-chip %CVs, inter-instrument %CVs of HDL-2b were less than 10%, which was the set acceptance criteria. With 243 samples from healthy individuals demonstrated that the mean value of HDL-2b% is about 25% in both male and female.*

***Conclusions:*** *Our data demonstrated that the MFE system is a fast, automatic, and reliable system that can obtain the percentage of HDL-2b with high precision.*

## Introduction

CHigh-Density Lipoprotein (HDL) subfractions refer to different forms of HDL cholesterol in the bloodstream. HDL cholesterol used to be referred to as "good" cholesterol because it helps remove excess cholesterol from the body and plays a protective role in cardiovascular health. In-depth study, however, had identified controversial functions between the two primary subfractions HDL-2b and HDL3.

The larger, less dense particle HDL-2b particles are believed to be more efficient in reverse cholesterol transport (RCT), while the smaller and denser HDL3 particles is considered less effective in RCT compared to HDL2, may be associated with increased risk of cardiovascular events [1-3].

It's important to note that measuring HDL subfractions is not routinely performed in standard clinical practice in most clinical organizations, mainly due to the lack of knowledge for their clinical significance [4]. Also, the tedious procedure and high cost of the assay are factors the hinder the clinical application of the assay. We have developed a unique microfluidic electrophoretic system (MFE) using microfluidic electrophoretic technique in combination with sophisticated identification method that can complete an entire testing procedure within one hour. The verification result from this paper demonstrated the MFE as the most advanced technology in HDL subfrationation.

## Materials and methods

### Materials

#### Equipment and testing kit

MICEP-30 microfluidic chip analyzer and HDL subfractioning kit are developed by Ardent Biomed.

#### Blood sample

The study was approved by the ethics committee of the related hospitals and all subjects were provided with written informed consent. Blood samples were collected into the additive-free vacuum tube, allowed 30-60 minutes for coagulation. The samples were then centrifuged at 3000 rpm for 10 minutes, and serum was stored at -80º C before analysis. Sample preparation was performed according to the instruction

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of HDL subfractioning kit (Ardent biomed, China) prior to loading onto the MICEP-30 Analyzer.

## Methods

### MFE Procedure

Operation procedure follows the instruction provided by the manufacturer. Briefly, 2 ul serum from each sample was mixed with 98 ul of dilution buffer, and 7 ul was loaded onto the chip. A quality control was also loaded with the samples. Transfer the chip onto the stage on the MIEP-30 analyzer.

### GE procedure

Separation of serum HDL particles was performed by non-denaturing polyacrylamide gradient gel (3-31%) electrophoresis (GGE) [5]. After electrophoretic separation, the gel was scanned to examine lipoprotein subclasses using the Image Scanner (Amersham Pharmacia Biotech, Vienna, Austria) with Image Quant software (version 5.2; 1999; Molecular Dynamics, Sunnyvale, USA). The dominant HDL particle diameter was defined by estimating the diameter of the major peak in their regions in the densitogram. The relative proportions of HDL-2b were estimated by determining the areas under the peaks after densitometric scans of the samples. Procedure for determining HDL subclasses was done according to Rainwater et al [6].

## Methods

### The statistical analysis

Data were expressed as mean ± standard deviation. SPSS12.0 program was used in statistical computing. Following calculations were performed:

$$SD_{chip} = \sqrt{SD_{rm}^{2} - \frac{OR^{2}}{N}}$$

$$SD_{Total} = \sqrt{SD_{rm}^{2} + \left(\left(\frac{N-1}{N}\right) \times OR^{2}\right)}$$

$$CV_{r} = \frac{SD_{chip}}{\bar{X}}$$

$$CV_{total} = \frac{SD_{T}}{\bar{X}}$$

where:

$SD_{chip}$ = observed inter – chip standard deviation

$SD_{rm}$ = standard deviation of run means

$OR$ = observed repeatability

$SD_{T}$ = observed total standard deviation

$N$ = number of replicate analyses per run

$\bar{X}$ = average of all results

## Results

### Correlation of MFE with GGE

GGE method has been used as a traditional standard for accuracy study of lipid subfractions [7]. As there are no known HDL-2b% standards or methods against which to establish true reference results, the relationship between the MFE and the recognized GGE method provides a convincing evidence for the accuracy of the MFE system.

The study employed 99 serum specimens that were collected from volunteers who provided informed consent. MFE and GGE were performed by separate individuals and the results were summarized by a third person. The study was conducted with one lot of HDL subfractioning kits, one lot of chips, and three different instruments of MICEP-30 analyzer.

The correlation between the MFE system and GGE was determined to have a linear relationship with an R of 0.64 for HDL-2b % values. While the R was less than the proposed limit of 0.8, it is recognized that the two methods are different in many aspects, and a linear relationship meets expectations. Also, when the HDL-2b% values were converted to HDL-2b (mg/dL) values so that the breadth of results were maximized, R increased to 0.93 (Figure 1).

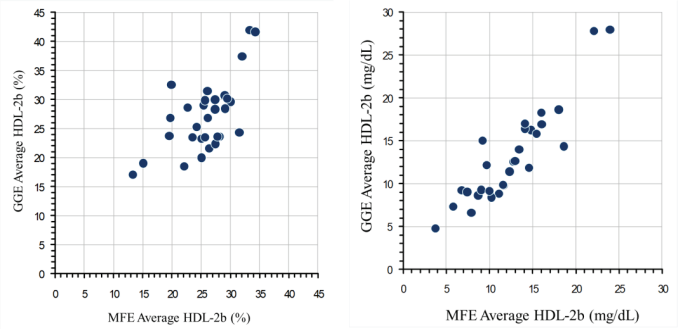


***Figure 1.*** *Correlation of MFE and GGE methods in detection HDL subfraction by percentage (A) and by concentration (B) Ninety nine serum samples were assayed in parallel with MFE method and GGE method spontaneously. The observed results were calculated into percentage and concentration of HDL-2b were presented and plotted. A linear correlation was found in both of the of the plot and statistical significance was obtained while expressed in concentration (R=0.93)*

### Internal precision

Three serum samples were identified and defined as LOW, MIDDLE, and HIGH, targeted at HDL-2b% levels of 12%, 20%, and 35%, respectively. Each sample was assayed in triplicate on one chip twice a day, with one run in the morning and the second run in the afternoon, for 20 days. The study included one lot of reagents, one lot of chips, one MICEP-30 Analyzer, and two operators (one morning operator and one afternoon operator). The result was summarized in table 1.

The study demonstrated that intra-chip %CVs were less than 10% for all three samples, the inter-chip %CVs were less than 15% and 10% for samples below and at or above 12 HDL-2b%, respectively, and the total precision %CVs were less than 15% for all three samples. Further, for the three samples, the within-chip %CV ranged from 1.6% to 6.4%, the between-run %CV ranged from 2.3% to 4.0%, and the between-day %CV ranged from 1.0% to 1.6%.

***Table 1**. Internal precision assay for intra-chip and inter-chip using 3 standard samples designed as LOW (12%), MIDDLE (20%) and HIGH (35%) of HDL-2b.*

| | LOW | MIDDLE | HIGH |
|---|---|---|---|
| *Mean HDL-2b% | 12.6 | 20.8 | 35.2 |
| Intra-chip CV | 6.4% | 2.4% | 1.6% |
| Inter-chip CV | 4.3% | 2.5% | 2.8% |
| $CV_T$ | 7.7% | 3.5% | 3.2% |

*Mean HDL-2b%: average of triplicated sample on one chip twice a day for 20 days; Intra-chip CV: coefficient of variation within one chip; inter-chip CV: coefficient of variation among chips; $CV_T$ : coefficient of variation of collective data.

## External precision

The study was conducted at three sites including Ardent Biomed Lab (site I), Gaozhou People Hospital (site II), and the Secondary Hospital of Guangzhou (site III). All three samples, LOW, MIDDLE, and HIGH, were assayed in triplicate on a single chip four times a day for five days at each site. The study included three lots of HDL subfractioning kits, three lots of chips, and three different instruments. Precision was estimated for inter-chip performance, inter-instrument performance, and total (overall) performance. The study demonstrated that at all three sites, all specifications were met. The intra-chip %CVs were less than 10% for all three samples, the inter-chip %CVs were less than 15% and 10% for samples below and at or above 12 HDL 2b%, respectively, and the total precision %CVs were less than 15% for all three samples. (Table 2)

***Table 2.** External precision assay for intra-chip and inter-chip using 3 standard samples designed as LOW (12%), MIDDLE (20%) and HIGH (35%) of HDL-2b from 3 testing centers.*

| | LOW | | | MIDDLE | | | HIGH | | |
|---|---|---|---|---|---|---|---|---|---|
| Site | I | II | III | I | II | III | I | II | III |
| *Mean | 12.6 | 12.7 | 12.0 | 19.8 | 20.7 | 19.2 | 32.3 | 30.6 | 33.8 |
| SDrm | 0.42 | 0.65 | 0.81 | 0.78 | 0.50 | 0.76 | 0.98 | 1.17 | 1.26 |
| $S_E$ | 0.53 | 0.47 | 0.33 | 0.55 | 0.48 | 0.67 | 0.50 | 0.74 | 0.82 |
| $S_C$ | 0.75 | 0.59 | 0.37 | 0.39 | 0.70 | 0.67 | 1.22 | 1.09 | 0.86 |
| $S_T$ | 0.91 | 0.75 | 0.50 | 0.67 | 0.85 | 0.95 | 1.32 | 1.32 | 1.19 |
| $CV_E$ | 4.20% | 3.70% | 2.80% | 2.80% | 2.30% | 3.50% | 1.60% | 2.40% | 2.40% |
| $CV_C$ | 5.90% | 4.60% | 3.10% | 2.00% | 3.40% | 3.50% | 3.80% | 3.60% | 2.50% |
| $CV_T$ | 7.30% | 5.90% | 4.20% | 3.40% | 4.10% | 5.00% | 4.10% | 4.30% | 3.50% |

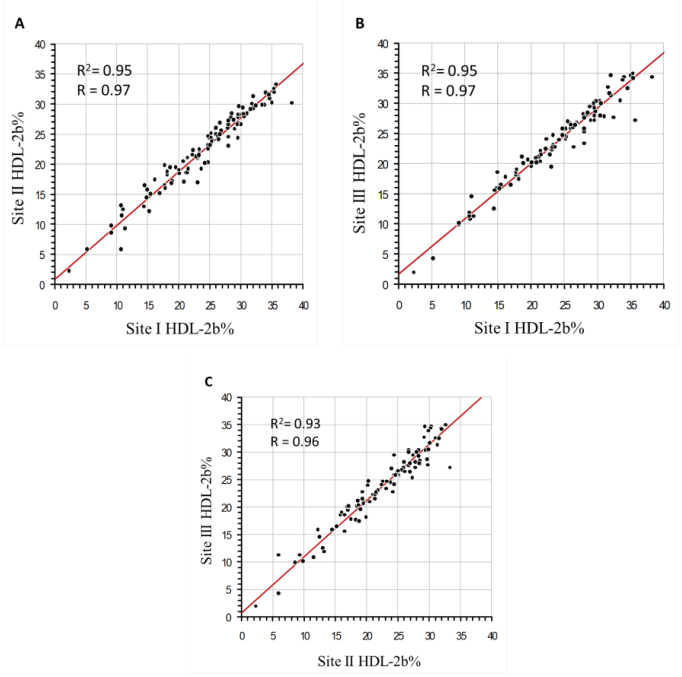


***Figure 2.** Variations among technical operators in three testing labs were investigated using 99 samples. All samples were tested in each of the three labs by multiple individual operators in a blinded manner. Comparison were performed between each site (site I vs. site II (A); site I vs. site III (B); and site II vs. site III (C)).*

Operational difference among technicians from the three sites were compared with 99 samples were tested in each site in a blinded manner. Three comparative sets of results (Site 1 vs. 2, Site 1 vs. 3, and Site 2 vs. 3) were analyzed for correlation coefficients (Figure 2). The various operators were assigned for the HDL-2b% assay using MFE system for all 99 samples, and collective data were plotted in figure 2. The R value of site I against site II is 0.97, and R2=0.95. Same result was found by site I against site III. The R value of site II against site III is 0.96, while R2 = 0.93 (Figure 2).

## Reference range determination oncluding remarks

Two hundred and forty three serum samples from presumptively healthy individuals who were eligible as blood donors were tested. The samples were assayed in groups of ten on a single chip, and three repeats were conducted. The results were partitioned by gender and age.

The average age of females is 36 ranging from 18 to 62, while the average age of males is 39 ranging from 18 to 65. The mean values, the 2.5 and 97.5 percentiles of the male and female sampling distributions were calculated and presented in Table 3. Mean values of both males and females were almost identical, close to 25, and there is no significant difference of minima, maxima, and mean between the genders (P>0.05).

***Table 3.*** *Reference range of HDL-2b% determined by 243 presumptively healthy individuals.*

| | Females | | Males | |
|---|---|---|---|---|
| | **Age** | **HDL-2b%** | **Age** | **HDL-2b%** |
| N | | 123 | | 120 |
| Minimum | 17 | 10 | 18 | 3.2 |
| Mean | 36 | 25.3 | 39 | 25.4 |
| Maximum | 62 | 38.4 | 72 | 38.2 |
| 2.5th Percentile | | 14.4 | | 12.8 |
| 97.5th Percentile | | 34 | | 34.5 |

## Discussion

While HDL cholesterol levels as a whole are important for assessing cardiovascular health, understanding the specific subfractions, such as HDL-2b, can provide additional information [8]. The clinical measurement of HDL-2b levels may provide insights into an individual's lipid profile and cardiovascular risk, and significantly influence the diagnosis and treatment of certain diseases [9, 10]. HDL-2b particles are considered large, buoyant, and thought to have stronger cardioprotective properties [11,12]. Low levels of HDL-2b may be associated with an increased risk of cardiovascular disease. It is worth noting that research on HDL-2b has specifically been limited compared to total HDL cholesterol or other lipid parameters [13]. Therefore, the clinical interpretation of low HDL-2b level should be considered in conjunction with other lipid markers and individual cardiovascular risk factors.

The principle of microfluidics is based on the manipulation and control of small volume of fluids (typically in the range of microliters or nanoliters) in microscale channels or devices [14]. Microfluidics leverages the unique behavior of fluids at the microscale, where surface forces dominate over inertia, to enable precise and efficient handling of fluids [15].

We developed the first HDL subfractioning system using the combination of microfluidic electrophoretic device in combination with fluorescent detectors that can precisely differentiate subfractions by size and position, with a sophisticated computing system to calculate the percentage of each subfraction. When verified by GGE method, a linear relationship with a correlation coefficient of 0.64 was obtained. This low value for the correlation coefficient was largely attributable to the cluster of results around the value of 25 HDL-2b%. In order to “spread out the data” as is needed for meaningful regression statistics, the results were converted to mg/dL, and then a correlation coefficient of 0.93 was obtained.

The verification assays on the MFE system in both internal precision and external precision proved its stability amongst chips, instruments, testing centers, as well as operators. Our data demonstrated that MFE is a well-developed automatic system that works fast and accurate, and the only system that has been approved by authority for clinical applications.

The separation of HDL subfractions can be achieved using various laboratory techniques, each with its advantages and limitations [16]. The choice of technique depends on the specific research or clinical objectives, available resources, and expertise. Here are some commonly used techniques for HDL subfraction separation. Ultracentrifugation is considered the gold standard technique for HDL subfractionation. Ultracentrifugation provides excellent resolution but requires specialized equipment and technical expertise [17]. GGE offers good resolution but may not provide as detailed subfractionation as ultracentrifugation, and requires specific equipment and expertise for sample preparation and interpretation [18, 19]. Another commonly used method is precipitation. Various precipitation methods, such as heparin-MnCl2 precipitation, dextran sulfate-MgCl2 precipitation, or phosphotungstate-Mg2+ precipitation, have been used to separate HDL subfractions. Precipitation methods are relatively simple and cost-effective but may provide less detailed subfractionation compared to ultracentrifugation or electrophoresis [20, 21]. Nuclear Magnetic Resonance (NMR) Spectroscopy provides information on the overall size distribution of HDL particles rather than specific subfractions. NMR spectroscopy is a non-invasive and high-throughput technique but may not provide detailed subfractionation like other methods [22].

It's important to note that each technique described above has its advantages and limitations in terms of resolution, cost, complexity, and availability [5]. The MFE system has the superior advantages amongst all the available methods that deemed as the fastest, most efficient and cost-effective method.

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