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# External Quality Assessment Program on CD4+ T-Lymphocyte Counts for Persons with HIV/AIDS in Thailand: History and Accomplishments

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**SUMMARY** A CD4 count External Quality Assessment (EQA) program is important for the clinical monitoring of persons infected with HIV/AIDS. The purpose of the present study was to evaluate the CD4 EQA performance program of the flow cytometer laboratories that perform routine CD4 counts for these patients in Thailand. Stabilized whole blood samples were sent to participating laboratories to determine the percentage and absolute counts of CD4+ T-lymphocytes using their routine procedures. The data were analyzed and reports sent to the participants within one month. Most participating laboratories produced results that were within two standard deviations (SD) of the mean, while the average inter-laboratory coefficients of variation were less than 8% for CD4+ T-lymphocytes. This program was found to improve the reliability of CD4+ T-lymphocyte determinations. This test is becoming increasingly important as Thailand and other Southeast Asian countries scale up their national programs that provide access to antiretroviral therapy for persons living with HIV/AIDS.

The Thailand National AIDS Program (NAP) is responsible for at least 100,000 HIV-infected people among the national population of 65 million. New annual HIV infections decreased from 17,000 in 2004 to around 14,000 in 2007.[1] However, total HIV infections are currently at least 700,000.[2]

CD4+ T-lymphocytes are a primary target of HIV and are preferentially depleted throughout the course of the disease.[3] Thus, they are markers for both the prognosis and therapeutic monitoring of the disease. Whole blood CD4+T-lymphocyte levels are measured using flow cytometric immunophenotyping. This is widely accepted as the standard method for this purpose due to its superior accuracy, precision and reproducibility.[4]

The use or misuse of CD4+T-lymphocyte measurements has a crucial impact on the effective management of HIV/AIDS patients. It is essential that accurate daily Internal Quality Control (IQC) and proficiency testing or External Quality Assess-

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ment (EQA) programs are employed to ensure reliable results. Satisfactory performances in CD4+T-lymphocyte EQA are recommended for HIV research and clinical trial programs within many parts of the world, including Thailand.

The limited budget for the Antiretroviral Therapy (ART) Program has hampered the EQA program in Thailand. Previously, the Thai government requested funding from the Global Fund to Fight AIDS, TB and Malaria for ART care and treatment, but this funding was not sufficient to cover all cases of the disease. Consequently, many infected people did not have access to the NAP to follow up their CD4+T-lymphocyte measurements. This testing is an important monitoring tool used in HIV treatment in Thailand. An alternative measurement, viral load testing, is far more expensive. Newly-infected patients also should be introduced to NAP and tested for their CD4 baseline values, so that these and other infected persons receive ART and follow-up CD4+ T-lymphocyte count determinations.

CD4+ T-lymphocyte counts are performed by flow cytometry laboratories located in public health hospitals throughout the country. Sixteen flow cytometers were purchased by the Ministry of Public Health in 1991, and there are currently over 150 such machines performing routine CD4+T-lymphocyte count tests. All regional, provincial and some large district hospitals provide testing services for nearby hospitals that lack this equipment. More than half the flow cytometers in the country can perform the dual platform (DP) technique, although the single platform (SP) technique is also commonly employed.[5] SP is a simplified flow cytometer which utilizes a single instrument.[3,5] It provides an absolute CD4 values with less variation than the DP method. The DP technique uses two instruments: a flow cytometer for determining the percent CD4+T-lymphocytes among all lymphocytes, and a hematologic cell analyzer which determines absolute lymphocyte numbers, and hence the measurements show more variation than the SP approach. The only SP flow cytometer used in the country is the FACSCount. The original 16 flow cytometers were bought from Beckton Dickinson Biosciences (BDB, San Jose, CA, USA) for the DP technique. Later, the Ministry received additional funding and subsequently purchased both DP and SP machines. Thailand presently has approximately 150 flow cytometers. The DP machines account for 100 of these while the remaining 50 use the SP technique (FACSCount).

The Center of Excellence for Flow Cytometry is the sole CD4 EQA provider in Thailand and participate in two international CD4 EQA programs: Quality Assessment and Standardization for Immonulogical Measures Relevant to HIV/AIDS (QASI, Canada-based program) and United Kingdom National Quality Assessment Services for Lymphocyte Immunophenotyping (UKNEQAS).[6,7] The QASI panel is shipped three times a year and free of charge, while the UKNEQAS annual fee and shipment charges are relatively expensive. UKNEQAS panels are delivered six times a year. For the past few years, the quality of the UKNEQAS samples was substandard, possibly as a result of temperature differences between the two countries. These samples necessitated the use of the lyse-and-wash procedure not usually performed routinely.

The CD4 EQA program has two main objectives. Firstly, it must conduct a low-cost national EQA and standardization of flow cytometer CD4+T-lymphocyte determinations in HIV/AIDS patients. Secondly, the EQA provider should assist in the country's flow cytometer laboratory performance evaluations, and incorporate scientifically and educationally-based schemes in order to monitor their improvement. Towards the middle of 2008, there were 30 EQA trials with 130 participating laboratories mostly from Ministry of Public Health. In this report, we summarize and discuss the history and the findings from the last 6 years of our EQA program on CD4+ T-lymphocyte counts.

## MATERIALS AND METHODS

Aliquots of the same batch of stabilized EQA whole blood were distributed to participating laboratories throughout the country. The EQA blood sample panels were prepared from healthy donors' blood and stabilized according to a fixative method developed at the Center of Excellence for Flow Cytometry. Each batch of EQA blood was evaluated to ensure that immunophenotypic characteristics have been maintained during handling and transportation. Laboratories were instructed to acquire and analyze the EQA blood samples using their standard

operating procedure, which are normally followed the manufacturer's instructions.

A shipping schedule is sent annually. Six whole-blood trials are conducted per year. The first CD4 EQA trial is labeled Trial 01. Each trial consists of different panels based according to the number of participating members. As of mid-2008, the program had reached Trial 30. The panels were sent by post at ambient temperature within 1-3 days and then stored at 2-8°C until testing require. Four weeks were permitted for the laboratories to conduct tests and to return the results to the program provider.

After the participating laboratories received an EQA tube along with brief instructions and a blank report form, they recorded the date received and the integrity of the sample. Flow cytometer laboratories performed the CD4+ T-lymphocyte testing either by a lyse-and-wash procedure, whereas other laboratories used a lyse-no-wash procedure. The samples were routinely stained with three-color and two-color monoclonal antibodies for the DP and SP techniques respectively. Laboratories using SP technique were asked to report the absolute CD4+T-lymphocyte count while laboratories using DP technique were required to obtain a complete blood count using a hematology analyzer followed by %CD4+T-lymphocyte measurement. Both the percentage and absolute counts of CD4+T-lymphocytes were often determined by DP users. Most flow cytometer laboratories use automatic lymphocyte gating and appropriate BDB or other software to analyze the data. Submitted results not only included CD4+T-lymphocytes, but also CD3+ pan T-lymphocytes and CD8+ suppressor T-lymphocytes were also accepted and analyzed but only CD4+ T-lymphocytes are reported here.

The statistical analysis of the most important parameter (CD4+T-lymphocytes) was analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA). *P*-values (< 0.05) were calculated for the response rates of both platforms to show the statistical significance of differences by chi-square. The mean and standard deviation (SD) were used to group the ranges of CD4+T-lymphocyte values and percentage of coefficient of variation (%CV) for both DP and SP techniques. Laboratories with results > 2SD from the pool mean results were considered as outliers and were removed. The trimmed data were reanalyzed to obtain a trimmed mean and a SD for CD4+T-lymphocyte values. Each laboratory result was compared directly to the trimmed SD value. Data of mean ± SD for each individual trial were depicted using the Levy-Jennings plot. Performance assessment expressed by the average %CV from Trails 12 to 30 for all participating SP laboratories and Trials 1 to 30 for all participating DP laboratories were also demonstrated by longitudinal plots.

## RESULTS

Thirty CD4 EQA trials were delivered to participating laboratories between 2002 and 2008. The results were returned on a monthly basis as required by the accompanying leaflet for each panel. Data was collected and analyzed using spreadsheet software.

Since SP protocols became available only 3 years ago, DP data was acquired exclusively for the initial Trials 01 to 11. The overall response rates for both platforms were high throughout the course of the trials (2002-2008). During the first year of SP availability, there was no significant difference in the response rates for each platform. However, in the next three years there were significant differences, as SP had a low response rate of 84%. This may be because some laboratories with access to both SP and DP cytometry did not use both methods due to time and budget constraints. Also, they may not use both platforms routinely, or in some cases only one machine was accessed as a backup in busy hospital laboratories or medical institutes. Furthermore, the DP flow cytometers were originally bought and distributed initially, whereas SP flow cytometers were purchased at a later time.

A total of 2,307 samples were sent to SP and DP users. The absolute and %CD4+T-lymphocytes values were both within the mean ± 2SD for 2,209 of these samples. Table 1 indicates significant differences between the CD4 EQA value distributions for both platforms. However, more than 70% of these were within the mean ± 1SD.

The average coefficient of variation (%CV) of SP for 19 trials (Trials 12 to 30) was 5.6%. The %CV was consistent (Fig. 1A) but two individu-

**Table 1** CD4 EQA value distribution and platform technique

| Results | Both platforms | Single platform | Dual platform |
|---|---|---|---|
| **No. samples** | 2,307 | 673 (29.2%) | 1,634 (70.8%) |
| **No. trials** | 30 | 19 (63.3%) | 30 (100.0%) |
| **No. sites** | 130 | 49 (55.7%) | 81 (92.1%) |
| **Mean values ± SD** | - | 468.2 ± 194.1 (absolute CD4 cells/$mm^3$) | 35.9 ± 10.1 (% CD4) |
| **No. samples within SD groups** | | | |
| **a) Within ± 1SD** | 1,656 (71.8) | 561 (83.4) | 1,095 (67.0) |
| **b) Between ± 1SD and ± 2SD** | 553 (24.0) | 52 (7.7) | 501 (30.7) |
| **c) Outside ± 2SD** | 98 (4.2) | 60 (8.9) | 38 (2.3) |
| ***P-value*: a) *vs*. b) and c)** | < 0.001 | < 0.001 | < 0.001 |

al trials showed high %CV, those being Trial 20 (12.8%) and Trial 22 (11.6%). Experimental pipetting errors during sample preparation for FACSCount[8] may account for this. For example, laboratory personnel may not have conducted tests using BDB-provided electronic pipettes. In addition, machines may not have been correctly calibrated. The results for the DP technique were inconsistent, showing a high %CV in the first four trials (Fig. 1B). The average %CV was 18.9%, but dropped to < 10 %CV after trail 5.

The results were analyzed and depicted using a Levy-Jennings plot. Fig. 2 illustrates EQA Trial 07 (% CD4+T-lymphocytes), which was conducted in 2003. Any outliers over ±2 SD from the mean were deemed unacceptable. Participants were coded on the X axis and new members were added each year, as shown on the X axis. The right hand scale shows the consensus mean of %CD4+T-lymphocytes ±1 SD and ±2 SD. The left hand scale shows the %CD4+T-lymphocytes. Fig. 3 illustrates EQA Trial 15, conducted in 2005, which indicates that laboratories participating for longer durations within the EQA program show an increased performance in these later trials.

## DISCUSSION

We separated the analysis into two platforms, single and dual. In the first 11 trials, the %CD4+T-lymphocytes were analyzed since the DP method was used exclusively. Later, both DP and SP protocols were used, producing both %CD4+T-lymphocyte and absolute CD4+T-lymphocyte data. The % value was required rather than absolute value analysis only, because up to two thirds of flow cytometer users performed the DP technique, thus automatically providing % CD4+T-lymphocyte values.

The SP technique yields more accurate absolute CD4 values than the DP technique.[8,11] This is due to the variations that occur with the use of hematology analyzers during the DP process.[6] However, following the development of new BDB reagents and software[12], BDB intends to upgrade all FACSCount software in Thailand so that both % CD4 values and absolute values can be acquired and printed concurrently. At present, however, Thailand will continue to use the DP technique as its conventional technology, unlike other countries such as Brazil or various African countries.[13,14] The DP protocol was the first one to be used in Thailand, and laboratory personnel are familiar in using its protocol. Moreover, approximately half the DP machines in the country contain auto-loading for high throughput laboratories. Although the SP method results in less inter-laboratory variability, the higher response rate of DP and declining response rate of SP (data not shown) is supportive of our prediction that DP will be preferable to SP. It should be noted that in the absence of DP technology, SP laboratories should maintain a rigorous testing of EQA panels when they received.

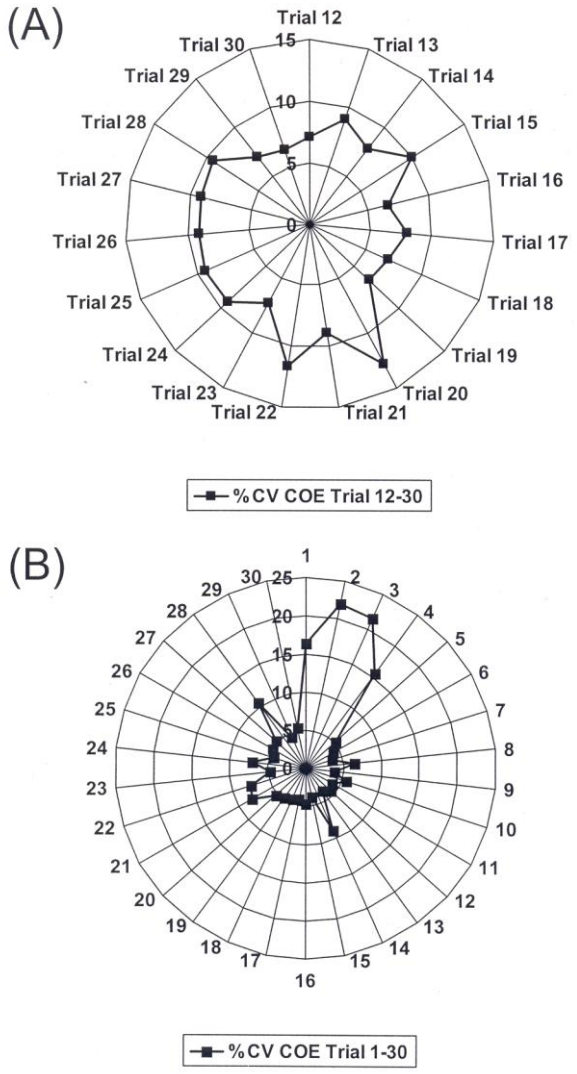


**Fig. 1** Longitudinal performance assessment of the coefficient of variation (%CV) of SP technique obtained from Trials 12 to 30 **(A)**; %CV of DP technique obtained from Trials 1 to 30 **(B).**

The %CV measurements for the SP technique in Trials 12-30 were low and quite consistent (Fig. 1A). However, two trials (Trials 20 and 22) yielded a high %CV, possible due to the aforementioned equipment error or because the means of the absolute CD4+T-lymphocyte values were the least (less than 300 cells/mm$^3$) among the trials. This is in accordance with previous studies[7,15,16] showing a high %CV when CD4+T-lymphocyte measurements were at low or medium levels. As mentioned previously, one third of the flow cytometers used in the country are SP machines (FACSCount), which contain one forward scatter feature along with PE and PE-Cy5 fluorochromes. The machines also do not serve high throughput laboratories and therefore tend to be used in small- and medium-sized laboratories. Laboratory personnel, who are medical technologists, usually conduct CD4+T-lymphocyte count testing and have more experience with the DP technique. They typically perform the test at specific times during the week (e.g. once or twice a week, or every second day) rather than daily due to other time and work commitments. The highest number of tests performed is approximately 1,000 per month at regional,

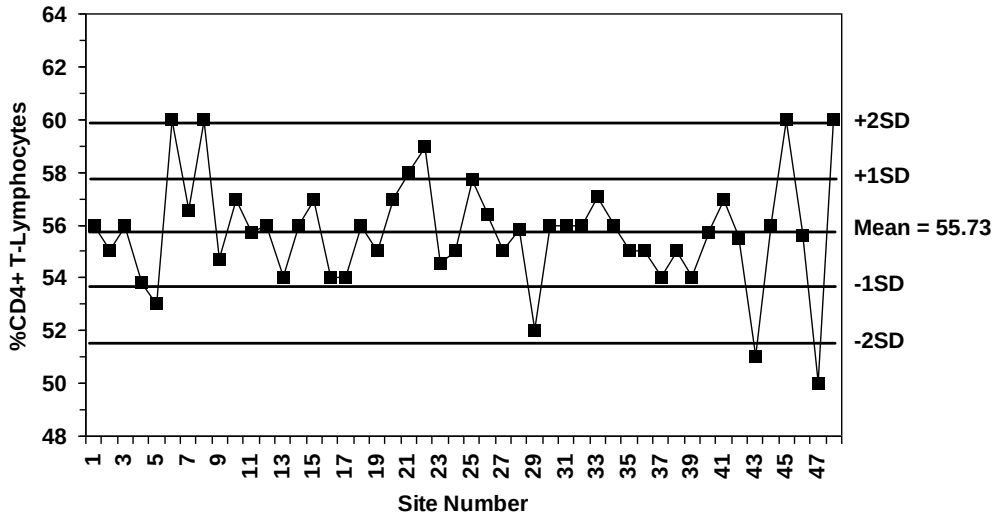


**Fig. 2** Levy-Jennings plot demonstrating % CD4+ T-lymphocytes obtained from Trial 7 in 2003.

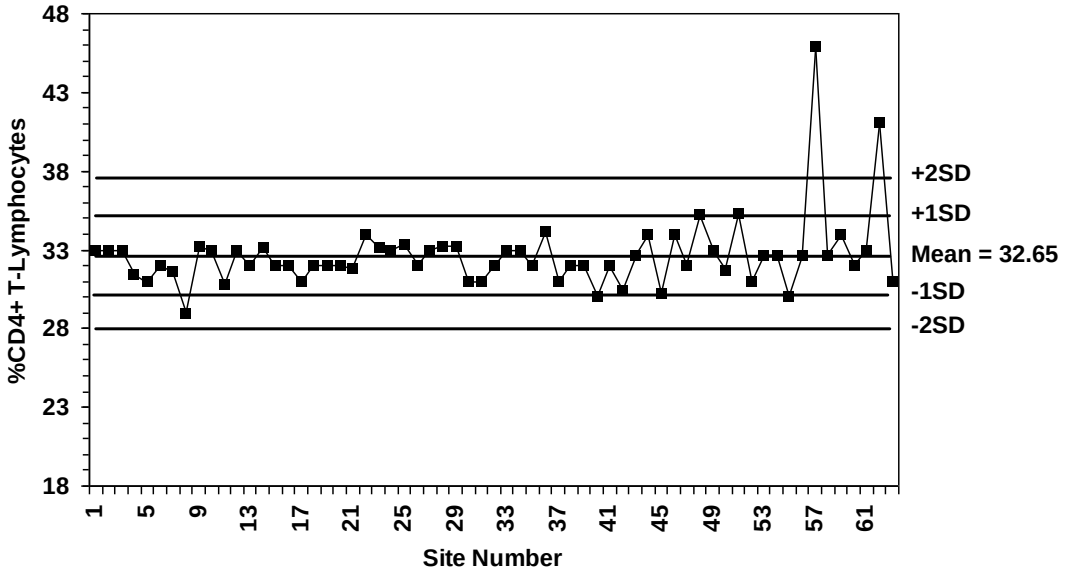


**Fig. 3** Levy-Jennings plot demonstrating % CD4+T-lymphocytes obtained from Trial 15 in 2005.

provincial or medical institute laboratories, far less than in African regional laboratories.[13]

Results in Fig. 1B show that the average %CV for CD4 EQA across Trials 01-04 from participating DP laboratories was high (18.9%) but later dropped to less than 10%. This may be because the program was relatively new to these participating laboratories at the beginning of this EQA program. Our results using this DP technique were similar to those of Malone *et al.*[9] (%CV range of 8.4% -23.0%) and Goguel *et al.*[10] (12.5%-14.5%). The average %CV of DP for the 30 trials was 7.2%, which was higher than the 19 SP trails (%CV of 5.6%) but lower than Malone *et al.*[9] and Goguel *et al.*[10] studies.

Fig. 2 shows that %CD4+T-lymphocyte mean value for EQA Trial 07 conducted in 2003 ex-

hibited a moderate level of fluctuation. While most values were within ± 2SD, some exceeded 2SD. In contrast to Trial 15 (2005) in which all the same individual laboratories (Laboratory No. 1 to Laboratory No. 48) performed exceedingly well with confined within 1 SD. It is reasoned that the longer the laboratories participating in the EQA trial, the better the performance of these laboratories.

Each year the EQA provider sends certificates of participation to members who are involved in all six trials. Participation is a requirement for any public health laboratory seeking certification for a national[17] or international[18] laboratory standard. In the near future, the EQA provider expects a new reporting system to be implemented. An internet-based reporting system is being developed that will allow all members to upload their results online. Furthermore, the designed report will be similar to laboratory test results acquired using other software, and which will be provided as longitudinal plots. This internet-based program is less complicated than other programs[19] and will also feature additional data for reagent tracking reports, including expiration date, lot number and manufacturer. The provider will generate a report to be returned to individual members within four weeks, thereby replacing the current manual report. The major benefit of this application is that it can display data in charts, and members can monitor their performance and compare their findings with other participants.

There are two EQA country programs directly supported by NAP funding. One is the HIV Serology EQA Program, provided by the National Institute of Health.[20] The other is this CD4 EQA program, which is provided by the center. The latter program has recently been integrated into the National Health Security Office Program and, as a result, the program provider has received some funding. Both programs have received national recognition throughout the medical profession.

There are some limitations to this program. Firstly, it reflects the performance of each participating laboratory in the analytical but not the pre- or post-analytical phases, since participating laboratories were aware that they were processing an EQA sample. Therefore, the interpretation of the program may not be direct and accurate indications of laboratory performance for the analysis of patient samples.[21] Some laboratories may perform well with EQA samples, but do so poorly using patient samples if they are not informed of the errors that can occur in pre- or post-analytical phases. Indeed, a blind EQA program might help improve routine testing. Unfortunately, no such program has yet been established in Thailand.

Secondly, there were a number of problems found during our evaluation. Although there were no complications with the lysis and lymphocyte gating procedures, some flow cytometers encountered technical problems arising from inconsistent maintenance issues or because participants did not perform calibration tests prior to sample runs, leading to inaccuracies. Our assistance was required in this regard when we were contacted by participants, or during our annual training meetings.

In conclusion, our CD4 EQA program using stabilized blood preparations improves the reliability of CD4+T-lymphocyte determinations. This is becoming increasingly important as Thailand scales up its national ART access program for persons living with HIV/AIDS. This national EQA program has arisen despite numerous challenges, requiring almost a decade to reach its present status.[22] It may be considered a significant advancement for Thailand as well as for the Association of Southeast Asian Nations (ASEAN), particularly as no such program had been available previously. Among its major roles are to provide adequate and ongoing information to its participants while evolving in an effort to meet participant satisfaction.[17,18] In addition, the program is currently expanding to neighboring ASEAN countries without incurring any major operating costs, thus assisting neighboring nations to implement the improvements that we have developed in our present study.

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