

## **Determination of Estrous Period in Female Rats (*Rattus norvegicus*) by Fourier Transform Infrared (FTIR) Spectroscopy Through Identification of Reproductive Hormone in Blood Samples**

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**Abstract:** Some methods in fertility study have been developed until now. One of the methods is determining estrous cycle by analyzing the concentration of reproductive hormones. Generally, hormone analysis method is destructive, difficult and expensive. Condition of fertility was analyzed by determining the concentration of progesterone using FTIR spectroscopy in this research. Blood samples were taken from ten rats during three estrous and non-estrous phases. The proportion of vaginal epithelium cells were qualitatively observed by vaginal smear to get the cycle phases determined. Blood samples were analyzed by FTIR, scanned in 400-4000  $\text{cm}^{-1}$ . RIA was used as a comparison method to analyze the samples. FTIR spectra were used to determined progesterone concentration in blood. Absorbance values of functional groups which represented progesterone such as ketone ( $1724 \text{ cm}^{-1}$ ), methyl ( $1375 \text{ cm}^{-1}$ ) and methyl-ketone ( $1354 \text{ cm}^{-1}$ ) were compared to hemoglobin's ( $1425 \text{ cm}^{-1}$ ) absorbance value which has relatively constant value during the cycles. RIA has confirmed progesterone concentration during the cycles. Estrous condition was achieved at level  $17,593 \pm 4,246 \text{ ng/ml}$  blood (FTIR relative absorbance to hemoglobin =  $0,853 \pm 0,310$ ). Non-estrous condition was achieved at level  $76.218 \pm 4.687 \text{ ng/ml}$  blood (FTIR relative absorbance to hemoglobin =  $1,024 \pm 0.268$ ).

**Key words:** Estrus cycle • FTIR • Functional groups • Progesterone • Rats

### **INTRODUCTION**

Species' survival depends on its reproduction success. One of the factors concerning the reproduction success is fertility. Species' fertility depends on the regulation and balance of reproductive hormones, such as estrogen and progesterone in female [1]. Fertility study is important to understand the variation of reproductive function of the species and its population [2]. By understanding that, conservation effort should be easy [3].

Enzyme immunoassay (EIA) and radioimmunoassay (RIA) are often used in fertility study. They are the most general methods used to measure the reproductive hormone concentration [4, 5]. Although the methods

could result accurate measurement, the kits are expensive and has expiration date. Moreover, the practice is not simple and use *destructive* radioisotopes [6]. Because of that, alternative method is needed.

Reproductive function in female is controlled by common hormonal system based on the hypothalamic control of the pituitary gonadotrophins, known as Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). FSH and LH act on ovary during reproductive cycle, either menstrual or estrous cycle. FSH brings follicles in ovary to be matured and dominant follicle will undergo ovulation. Ovulation will be initiated by LH. The more mature the follicle, the more estrogen hormone will secreted from follicle. After ovulation occur, the remainder follicle will become a corpus luteum, which secreted

progesterone hormone [7]. By indicating the secretion of progesterone in blood, we will know that all the process in one cycle should be done properly. Progesterone hormone indicates that ovulation has occur as a resultante of the work of pituitary that is influenced by hypothalamus.

*Fourier Transform Infrared (FTIR) spectroscopy* is the alternative method used in this research. FTIR is a universal instrument which has been used to analyze organic/inorganic samples. Its major advantages are accurate, safe, fast and sensitive [8, 9]. FTIR has been used in many research, such as analyzing gallstones [10], analyzing blood samples from renal failure patients [11], measuring plasma protein contents in blood [12], determining milk quality [13], diagnosing breast cancer tumors [14] and measuring reproductive hormones in rats urine [15].

Working principle of FTIR is recognizing specific functional groups in compound. FTIR could recognize progesterone's functional groups such as ketone ( $=O$ ) and methyl ( $CH_3$ ). Every functional group will be scanned in specific wavenumber with different absorbance value. Ketone will be scanned in  $1724\text{ cm}^{-1}$ , methyl in  $1375\text{ cm}^{-1}$  and methyl-ketone in  $1354\text{ cm}^{-1}$ . Absorbance value from every functional group will be compared to hemoglobin's absorbance value. Hemoglobin is represented by carboxylic acid ( $=COOH$ ) in  $1425\text{ cm}^{-1}$  [8]. Progesterone fluctuation in blood will be used to analyze fertility during the cycle.

## MATERIALS AND METHODS

**Location and Research Subject:** Ten three-months-old female rats (*Rattus norvegicus*) of Sprague-Dawley strain were housed in the animal care facility of the Biology Department, Faculty of Mathematic and Natural Sciences, University of Indonesia. Every two rats were housed in a cage ( $30 \times 20 \times 10$ )  $\text{cm}^3$  with woodchip bedding. Room's temperature and humidity were controlled. Light schedule was 12 h light and 12 h dark (lights on at 6.00 am). Water and food were provided ad libitum. Animals were acclimated for two weeks or after the body weights were stable at 180-200 g. The body weights were recorded daily. All animals were identified by using picric acid on their head, ears, hands, abdomen, back, feet and buttock.

**Sampling Method and Data Analysis:** Estrous cycle phases were determined by vaginal smear. Daily vaginal smear was taken at the same hour. Vaginal epithelium cells showed the phase of the cycle. Proestrus' major

population is nucleated cells; estrus is cornified cells; metestrus is nucleated cells, cornified cells and leukocytes; diestrus is leukocytes [16 -18]. Cell population always changes from time to time, under the influence of reproductive hormones [19].

Blood samples were collected during 3 (three) cycles from all females. Estrous blood samples were taken at estrous phases and non-estrous blood samples were taken at diestrous phases. All females were anesthetized and then bled from each tail using surgical scissors [20]. A 1.5 ml peripheral whole blood sample was collected from each female into a tube containing EDTA for FTIR and RIA analysis. Blood samples then were preserved in refrigerator at  $8^\circ\text{C}$ .

Blood samples were analyzed by FTIR type IR Prestige-21 [Shimadzu]. A 0.5  $\mu\text{l}$  blood sampel was smeared on window made of ZnSe to be scanned in  $400\text{-}4000\text{ cm}^{-1}$ . The presence of progesterone was identified by the peak of several functional group representing progesterone in specific wavenumbers. The progesterone levels in blood samples were obtained by measuring progesterone's functional group absorbance value relatively to hemoglobin absorbance value.

## RESULTS AND DISCUSSION

**FTIR Spectra:** Figures 1 and 2 show FTIR spectra from estrus and non-estrus blood samples. These spectra showed many peaks representing compounds' functional groups in blood. Pure progesterone spectra (Fig. 3) was used as a control. Similarity of the estrous and non-estrous spectra makes it difficult to observe functional group representing progesterone. Quantitative change of each peak is used to determine estrous and non-estrous condition. Data quantification was done by conversion of FTIR spectra to numerical by Microsoft Office Excel 2007.

### Functional Groups Determination

**Hemoglobin:** Hemoglobin consists of functional groups like methyl,  $=CH_2$  and carboxylic acid (Figure 4). These functional groups have their specific wavenumber in spectra. Methyl,  $=CH_2$  and carboxylic acid are located at  $1375\text{-}1382\text{ cm}^{-1}$ ,  $1640\text{-}1660\text{ cm}^{-1}$  and  $1396\text{-}1440\text{ cm}^{-1}$  respectively. Through the comparison of pure progesterone and blood spectra, carboxylic acid peaks were shown only in the spectra of blood sample. Peaks that represent  $=CH_2$  and methyl are not included in the analysis because these peaks were also shown in the spectra of pure progesterone and could cause bias in further analysis. Therefore, carboxylic acid functional

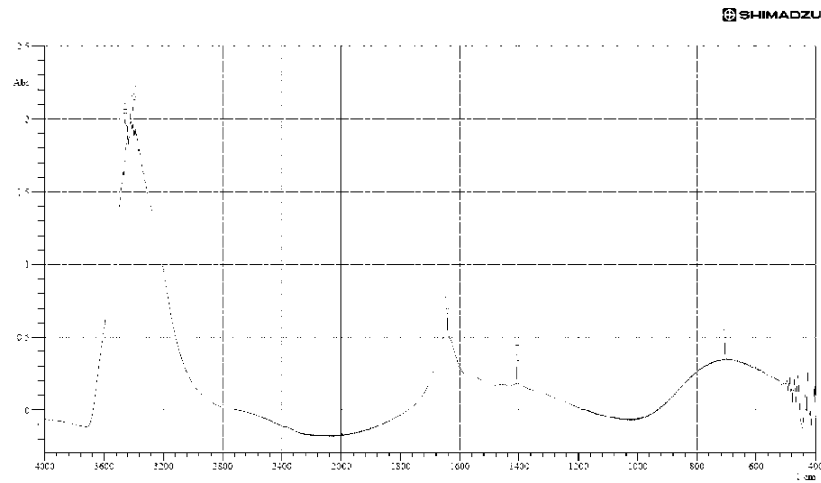


Fig. 1: Rat's blood spectra from estrus blood sample

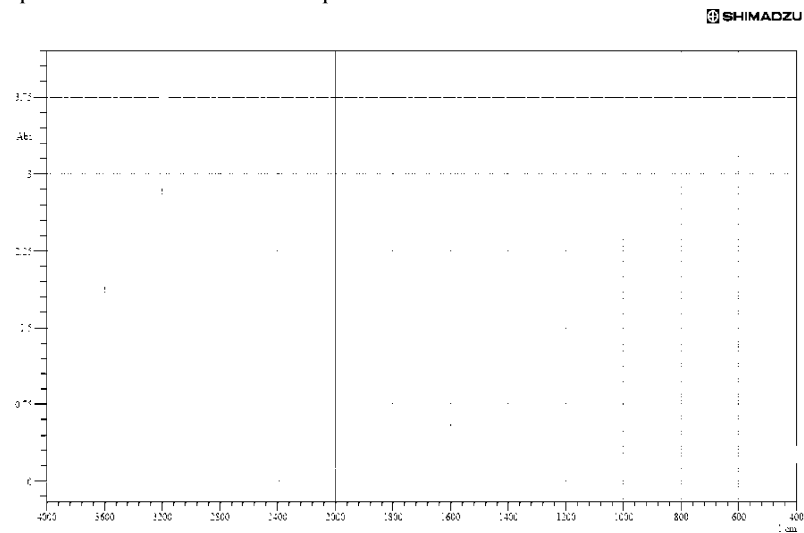


Fig. 2: Rat's blood spectra from non-estrus blood sample

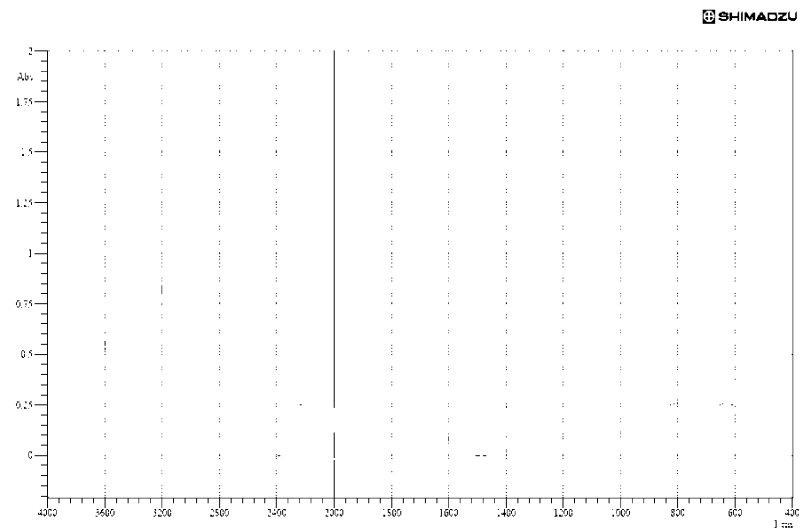
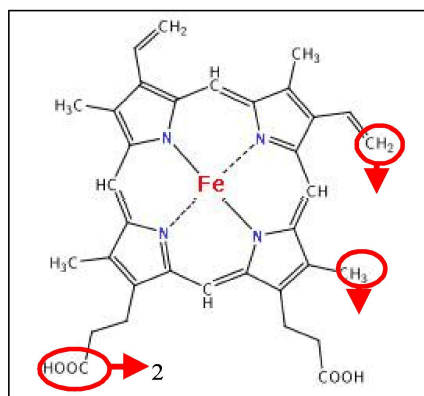


Fig. 3: Pure progesterone spectra



Notes: 1 :  $=CH_2$  2 : Carboxylic acid 3 : Methyl

Fig. 4: Hemoglobin chemical structure

Table 1: Hemoglobin absorbance value in  $1425\text{ cm}^{-1}$

| Estrus  |   | Non-estrus |   |
|---------|---|------------|---|
| Animal  | Absorbance value in $1425\text{ cm}^{-1}$ (%) | Animal     | Absorbance in $1425\text{ cm}^{-1}$ (%) |
| Rat1    | 0.164   | Rat1       | 0.280                                   |
| Rat2    | 0.273   | Rat2       | 0.201                                   |
| Rat3    | 0.311   | Rat3       | 0.265                                   |
| Rat4    | 0.290   | Rat4       | 0.264                                   |
| Rat5    | 0.201   | Rat5       | 0.270                                   |
| Rat6    | 0.253   | Rat6       | 0.240                                   |
| Rat7    | 0.218   | Rat7       | 0.241                                   |
| Rat8    | 0.184   | Rat8       | 0.342                                   |
| Rat9    | 0.157   | Rat9       | 0.220                                   |
| Rat10   | 0.246   | Rat10      | 0.263                                   |
| Average | 0.230   | Average    | 0.258                                   |
| SD      | 0.053   | SD         | 0.038                                   |

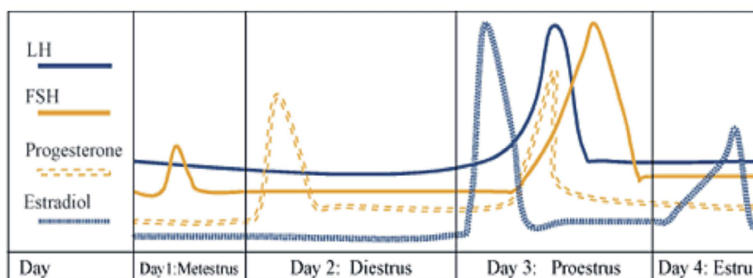


Fig. 5: Reproductive hormone fluctuation during one estrus cycle

group was used as hemoglobin marker [8]. Carboxylic acid at  $1425\text{ cm}^{-1}$  (Table 1) was used as hemoglobin marker because of its relatively constant absorbance average during estrus and non-estrus with the least standard deviation.

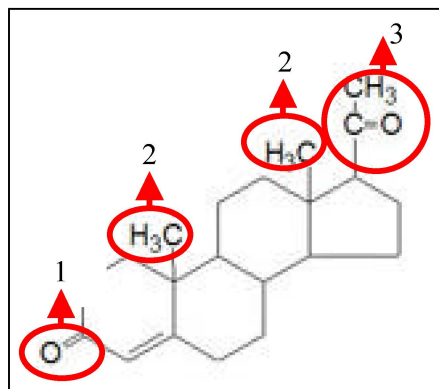
Hemoglobin was used as constant factor since its level is constant during estrous cycle and is not influenced by the fluctuation of the reproductive hormone during the cycle. Assuming that hemoglobin has relatively constant concentration during the cycles, therefore its absorbance value will remain constant during

the cycles. Progesterone functional groups' absorbance value was relatively measured to hemoglobin's absorbance value to obtain functional groups relative concentration.

**Progesterone:** Progesterone determination in blood is basically based on the concentration fluctuation during the cycles. Progesterone has higher concentration during non-estrous phase than estrus. Theoretically, reproductive hormone fluctuation during the cycle is showed in Figures 5.

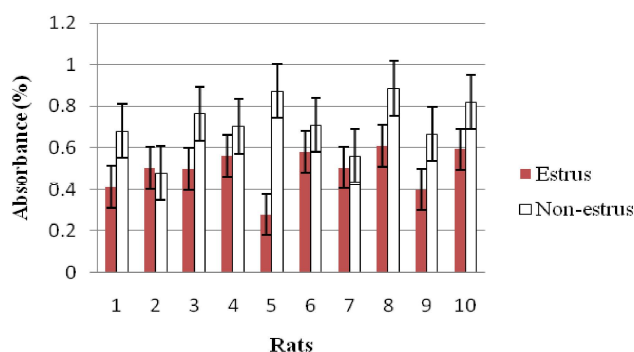
Table 2: Progesterone relative absorbance

| Animal  | Absorbance (%)                 |            |                                |            |                                       |            |
|---------|--------------------------------|------------|--------------------------------|------------|---------------------------------------|------------|
|         | 1724 $\text{cm}^{-1}$ (ketone) |            | 1375 $\text{cm}^{-1}$ (methyl) |            | 1354 $\text{cm}^{-1}$ (methyl-ketone) |            |
|         | Estrus                         | Non-estrus | Estrus                         | Non-estrus | Estrus                                | Non-estrus |
| Rat1    | 0.413                          | 0.680      | 0.715                          | 1.226      | 0.708                                 | 1.245      |
| Rat2    | 0.505                          | 0.479      | 1.197                          | 1.004      | 1.206                                 | 1.062      |
| Rat3    | 0.500                          | 0.765      | 1.326                          | 1.245      | 1.308                                 | 1.255      |
| Rat4    | 0.562                          | 0.703      | 1.261                          | 1.106      | 1.269                                 | 1.090      |
| Rat5    | 0.280                          | 0.874      | 0.954                          | 1.260      | 0.988                                 | 1.285      |
| Rat6    | 0.581                          | 0.710      | 1.069                          | 1.110      | 1.056                                 | 1.140      |
| Rat7    | 0.506                          | 0.561      | 1.060                          | 1.026      | 1.097                                 | 1.026      |
| Rat8    | 0.610                          | 0.886      | 0.878                          | 1.498      | 0.905                                 | 1.475      |
| Rat9    | 0.400                          | 0.666      | 0.710                          | 1.027      | 0.722                                 | 1.050      |
| Rat10   | 0.593                          | 0.819      | 1.091                          | 1.202      | 1.117                                 | 1.224      |
| Average | 0.495                          | 0.714      | 1.026                          | 1.171      | 1.038                                 | 1.186      |
| SD      | 0.104                          | 0.130      | 0.212                          | 0.150      | 0.209                                 | 0.139      |



Notes: 1: Ketone 2 : Methyl 3 : Methyl-ketone

Fig. 6: Progesterone chemical structure

Fig. 7: Ketone relative absorbance ( $1724 \text{ cm}^{-1}$ )

Progesterone consists of ketone ( $1707\text{-}1726 \text{ cm}^{-1}$ ), methyl ( $1375\text{-}1382 \text{ cm}^{-1}$ ) and methyl-ketone ( $1350\text{-}1369 \text{ cm}^{-1}$ ) functional groups (Figure 6). Within their spectral wave number ranges, these progesterone markers with the least standard deviation were chosen. Ketone was established at  $1724 \text{ cm}^{-1}$ , methyl at  $1375 \text{ cm}^{-1}$  and methyl-ketone at  $1354 \text{ cm}^{-1}$ .

Table 2 shows relative absorbance of several functional groups representing progesterone. The values

have been compared to hemoglobin absorbance value. Peak  $1724$ ,  $1375$  and  $1354 \text{ cm}^{-1}$  has lower absorbance average during estrus than non-estrus. Therefore, these peaks can be used as progesterone marker.

Figure 7 showed 90% of all females with ketone fluctuation pattern similar to progesterone fluctuation pattern during the cycle. Rat2 did not showed similar pattern due to the sampling period at late diestrus, exactly when progesterone level is low [19].

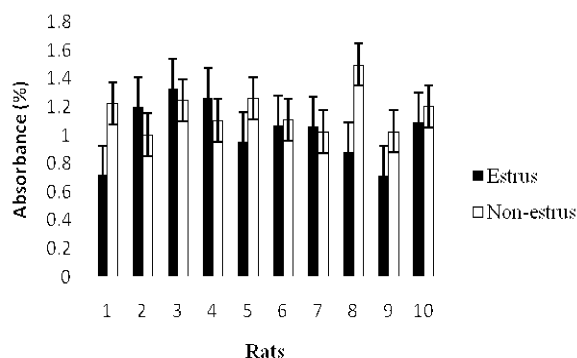


Fig. 8: Methyl relative absorbance ( $1375\text{ cm}^{-1}$ )

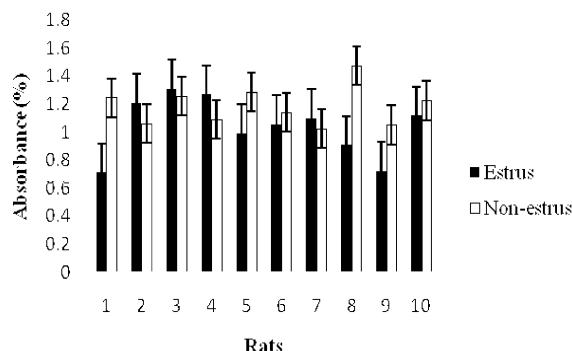


Fig. 9: Methyl-ketone relative absorbance ( $1354\text{ cm}^{-1}$ )

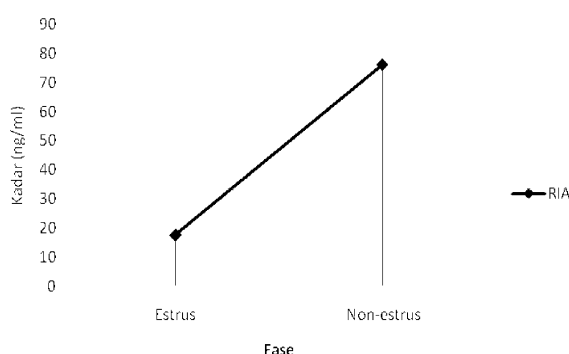
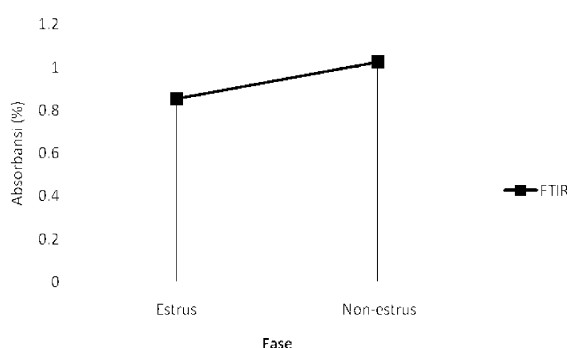


Fig. 10: Equivalence between FTIR relative absorbance and RIA concentration

Table 3: Equivalence between FTIR relative absorbance and RIA concentration

| Estrus                   |                    | Non-estrus               |                    |
|--------------------------|--------------------|--------------------------|--------------------|
| FTIR relative absorbance | RIA (ng/ml)        | FTIR relative absorbance | RIA (ng/ml)        |
| $0.853 \pm 0.310$        | $17.593 \pm 4.246$ | $1.024 \pm 0.268$        | $76.218 \pm 4.687$ |

Similarity between methyl ketone and progesterone fluctuation pattern also showed by 50% of all females (Figures 8 and 9). The other 50% did not showed similar pattern, due to the interference from other compounds which also have methyl and methyl-ketone functional groups [8]. Therefore, methyl and methyl-ketone were considered not strong enough to represent progesterone in blood.

Progesterone concentration has been verified by standard method in measuring hormone concentration, RIA. Result of RIA analysis showed progesterone concentration during estrus is  $17.593\text{ ng/ml}$  bloods and during non-estrus is  $76.218\text{ ng/ml}$  bloods. Table 3 and Figure 10 show hormone concentration in standard value and equivalence between FTIR relative absorbance and RIA concentration. FTIR relative absorbance values of  $0.853\%$  dan  $1.024\%$  were obtained from average measurement of Table 2. Based on Table 3.3,  $1\%$  of FTIR relative absorbance equivalent to  $20.625\text{ ng/ml}$  from RIA analysis.

Analysis result from FTIR and RIA show similar fluctuation pattern during cycle (Figure 10). FTIR peak absorbance can be measured directly, related to hormone concentration in blood [8] which also measured accurately by RIA. Both of the methods can measure reproductive hormone concentration accurately.

In conclusion, progesterone was recognized by function groups of ketone, methyl and methyl-ketone at  $1724\text{ cm}^{-1}$ ,  $1375\text{ cm}^{-1}$  and  $1354\text{ cm}^{-1}$  wavenumbers respectively. Estrus and non-estrus conditions were attained at  $17.593 \pm 4.246\text{ ng/ml}$  and  $76.218 \pm 4.687\text{ ng/ml}$  progesterone minimum concentration in blood.

## ACKNOWLEDGMENTS

This research is granted by Universitas Indonesia Multidiscipline Research Fund 2010. The Authors thank Mr. Rasyid, Alvin, Jajat, Ade Septian for technical assistance, Latifah Nurdahlanti for preparation manuscript in English and Dr. Dadang Kusmana for vaginal cytology method.

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