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RESEARCH ARTICLE

# Determination of Shelf Life of In Vitro Diagnostic Pregnancy Test by Accelerated Shelf Life Testing (ASLT)

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**ABSTRACT**

In Vitro Diagnostic (IVD) become the foundation for various things such as diagnosis, testing and monitoring in the health sector. One of them is the IVD hCG test to detect pregnancy. In the IVD there are components that bind antibody proteins therefore they are unstable to temperature changes. Changes in storage temperature can trigger changes in protein structure and function and interfere with binding to reagents, thus affecting detectability, shelf life and quality deterioration of the product. The purpose of this study was to determine the quality deterioration constant value per day and predict the shelf life of IVD pregnancy tests using the accelerated Arrhenius method by considering 3 temperatures. The samples in this study were In Vitro Diagnostic products in the form of pregnancy compact tests. The method used was accelerated shelf life testing (ASLT) with the Arrhenius equation. This study was conducted by storing samples at 3 extreme temperatures, specifically 40°C, 50°C, and 60°C for 35 days. The detection value of the IVD pregnancy test was read with the IC Reader every week. Based on the results, the constant value of deterioration is 0.0022 mAbs per day. The Arrhenius plot analysis followed first order kinetics. Shelf life calculations using the Arrhenius equation predicted that the shelf life of IVD pregnancy tests at room temperature 30°C is 1857.25 days, that is 5 years.

## INTRODUCTION

The existence of In Vitro Diagnostic (IVD) products is very beneficial for the community and healthcare professionals (Favaloro et al., 2011). IVD is used as a basis for diagnosing diseases, monitoring health, measuring physiological parameters, and even performing genetic identification (WHO, 2022). IVD tests with biological specimen samples can provide accurate results with relatively simple procedures, especially for IVDs dedicated to self-testing (Phillips, 2019). One of the IVDs available for self-testing is the hCG test for detecting pregnancy, produced by partner industries.

In Vitro Diagnostic Test hCG has a control line and a test line that bind antibody proteins. Antibody proteins are very sensitive to temperature. High temperatures can cause denaturation to the point of protein inactivation (Simon et al., 2018). High temperatures can alter the protein structure and result in the loss of the biological function of antibodies in recognizing the hCG antigen. Antibody proteins that cannot recognize hCG will affect the quality of the product. The quality of IVD test products can be observed from the detection values that need to be considered to determine the shelf life.

Shelf life has become the main challenge faced in ensuring product consistency. Determination of shelf life can be done using either real-time testing or accelerated testing approaches. The determination of shelf life for the hCG IVD test in the partner industry is currently only done using the real-time testing method. This approach takes a very long time because the monitoring of quality

degradation is assessed in real-time with normal storage. The determination of shelf life using the accelerated approach has been carried out by the Partner Industry, but it only considers one temperature, that is 60°C in the first production batch. The use of a single temperature in determining shelf life is considered less accurate. Based on WHO guidelines, the determination of shelf life through accelerated methods uses a minimum of 3 temperatures (WHO, 2022). Three different temperature levels can produce equations for determining shelf life more accurately. Therefore, accelerated testing using 3 different temperatures is necessary to meet WHO standards. Temperature is the main factor that needs to be considered in ensuring the accurate shelf life for IVD hCG test products using the accelerated method (Le Basle et al., 2020). The purpose of this research is to determine the quality degradation constant, specifically the detection value influenced by storage temperature. This research also aims to calculate the estimated shelf life of IVD pregnancy tests by considering 3 extreme temperatures in the prediction process.

Accelerated shelf life testing (ASLT) describes the simulation of accelerated environmental conditions that can trigger quality degradation, allowing the shelf life to be determined in a shorter time (S. Calligaris, 2019). The accelerated degradation of quality simulated on IVD test products was conducted with exposure to extreme temperatures. High temperatures can trigger changes in the structure and function of proteins and disrupt the binding of antibody proteins to reagents. The damage to antibody protein binding will affect detectability, leading to a decrease in the quality of IVD products. This study uses the ASLT method because it allows not only for the consideration of the effect of temperature on detectability but also for the efficient determination of shelf life (S. Calligaris, 2019). The selected temperatures are 40°C, 50°C, and 60°C because at these temperatures, the antibody structure will be disrupted but has not yet reached the antibody inactivation point, allowing for the observation of accelerated product quality degradation.

## LITERATURE REVIEW

### In Vitro Diagnostic Test

IVD has several constituent components, such as sample pad, conjugate pad, nitrocellulose pad, absorbent pad, and backing card. The sample pad is component that functions to absorb the specimen, filter out unwanted analytes, and deliver it to the conjugate pad. The conjugate pad is component that stores the reagent resulting from the coupling of color particles and hCG antibodies. This component aims to transfer the antigen that has bound to the conjugate reagent to the nitrocellulose pad (Millipore, 2013). The nitrocellulose pad is the component that serves as the membrane binding the control line and test line reagents. The control line reagent contains non-specific antibodies, while the test line reagent contains specific antibodies. The absorbent pad is a component that functions to absorb excess sample after passing through the test line and control line to prevent flowback. Backing card is a component used as the base for laminating other components to maintain the rigidity of the IVD test (Phillips, 2019).

The principle of the IVD pregnancy test is to detect the presence of the hCG hormone in the specimen. When the test strip is dropped with urine, the sample pad will absorb the urine and transfer it to the conjugate pad. In the conjugate pad, the antigen will bind with the conjugate reagent. In the nitrocellulose pad, the hCG antigen that has bound with the conjugate reagent will bind with the specific antibody on the test line. The antigen that does not bind to the test line will bind with the non-specific antibody on the control line. The remaining sample will then be transferred to the adsorbent pad. The interpretation of the pregnancy test strip results is that when there are two red lines (control and test line), it indicates a positive pregnancy result. If there is one red line (control line), it indicates a negative pregnancy result. Meanwhile, if there is one red line (test line) or no line appears at all, the test result is considered invalid and must be repeated (Dewanti & Anwar, 2022). The appearance of the line, in addition to being seen qualitatively by visual, can also be read quantitatively using an Immunochromatography Reader (IC Reader).

### Human chorionic gonadotropin (hCG)

Human chorionic gonadotropin (hCG) is a glycoprotein hormone that is often used to diagnose pregnancy. This hormone is primarily produced by the syncytiotrophoblast cells of the placenta to maintain pregnancy and is excreted through urine (Skogler et al., 2023). The level of hCG in the blood of pregnant women progressively increases during early pregnancy and can be detected in urine, so pregnancy tests using hCG detection can yield positive results in women who are pregnant (Lei et al., 2022). When pregnancy occurs, the level of the hCG hormone in the blood rises rapidly after implantation and peaks around weeks 9-11, which is the first trimester (Skogler et al., 2023).

### Shelf Life

Shelf life is the period of time when the product can be stored and still maintain its quality under specified storage conditions (Kim et al., 2022). There are two methods for determining shelf life, i.e. real-time and accelerated. Real-time shelf life testing refers to determining the shelf life based on changes in product quality under actual storage conditions. Meanwhile, accelerated shelf life testing refers to determining the shelf life based on changes in product quality under extreme storage conditions such as increased temperature (S. Calligaris, 2019). One of the accelerated methods is the Arrhenius equation. The Arrhenius method is generally used to estimate the shelf life of products whose deterioration is greatly influenced by temperature changes, triggering chemical reactions and contributing to product damage. In the IVD hCG test, the Arrhenius method was chosen based on the consideration that this method can link the rate of quality degradation with temperature.

## METHODOLOGY

### Tools and Materials

The tools used in this study include an oven, freezer, IC Reader (Hamamatsu C10066®), micropipette (Eppendorf®), yellow tip (OneMed®), microtube (OneMed®), microtube rack (Eppendorf®), logger device (HTC-1®), forceps, board rack, vortex (IKA-3®), beaker glass (Iwaki®), and measuring glass (Iwaki®). The materials used in this study include samples of In Vitro Diagnostic Test hCG products from Industry Partner, standard hCG reagent (Sigma Aldrich®) concentration 25 mIU, 70% alcohol.

### Research Variables

The variables used in this study are independent variables, controlled variables and dependent variables. The independent variable in this study was the storage temperature of in vitro diagnostic test products, that is 40°C, 50°C, and 60°C. The controlled variable in this study was the type of packaging with primary packaging in the form of cassettes and secondary packaging in the form of aluminum foil and paper boxes, storage time of 35 days. The dependent variable in this study was the detectability value and shelf life of in vitro diagnostic test products.

### Sample Preparation and Conditioning

The samples were pregnancy compact tests taken from a single batch of production with a total of 54 samples. The samples were grouped into 3 groups with 18 compact tests in each group. Each group was stored under extreme conditions that could accelerate the quality deterioration reaction. Group 1 was stored at 40°C ± 2°C, group 2 was stored at 50°C ± 2°C, and group 3 was stored at 60°C ± 2°C. The temperature was monitored and checked daily using a logger device. Each sample was placed in an oven in an aligned manner to avoid differences in temperature exposure of each sample and to avoid the weight influence on each other.

### Immunochromatography Reader Calibration

The calibration test was conducted to ensure that the Immunochromatography Reader (IC Reader) provided accurate measurements by comparing its results with a known standard. Therefore, this stage aims to ensure that the instrument can perform the expected functions according to the specifications. The IC Reader calibration test was carried out by checking the color signal intensity of the calibrator 6 times repetitively. The color signal intensities on the test line and control line of the

calibrator were 19.0 mAbs and 208.7 mAbs.

**Observation of Detectability**

The observation of detectability was carried out on storage day 0, 7, 14, 21, 28 and 35. The testing was done in triplicates at each storage temperature. The samples were dipped into microtubes containing 100uL of 25 mIU standard hCG reagent. After the reagent was absorbed and distributed, the control line and test line of the samples were observed in a horizontal position. Signal detection readings using the IC Reader were performed after the test line was visually apparent. The optimum reading was at minute 15.

**Data Analysis**

The data obtained from this study were the SD and RSD values from the IC Reader calibration, detection values from the detectability test and product shelf life time. The acceptance value of %RSD in the calibration is ≤2%. The observation data on the IC Reader calibrationc were interpreted descriptively through SD and RSD. The detectability IVD test results data in the form of detection values in mAbs units and time in day units were plotted and the linear regression equation was calculated, then the reaction order was determined by comparing $R^2$ at zero order and first order. Ln k was plotted against 1/T to obtain the regression equation, which was used to complete the Arrhenius equation. The Arrhenius equation is as follows:

$$k = Ae^{-\frac{Ea}{RT}}$$

$$\ln k = \ln A \left(-Ea/R\right)\left(1/T\right)$$

With the **k** is the quality deterioration constant, **A** is a constant independent of temperature, **Ea** is the activation energy, **T** is the temperature (Kelvin), and **R** is the gas constant (1.986 kal/mol) (Abdullah, 2016). The Arrhenius equation was then substituted with the desired storage temperature, specifically room temperature 30°C, to predict the shelf life calculated using Microsoft Excel 2021 software.

**Research Scheme**

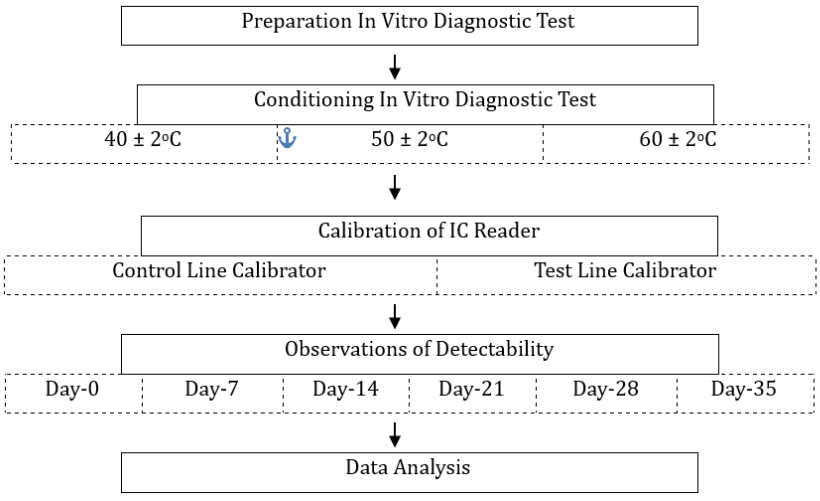


**Figure 1. Research Scheme**

## RESULTS AND DISCUSSION

### Preparation and Conditioning of In Vitro Diagnostic Tests

The preparation and conditioning of in vitro diagnostic tests as samples were carried out at

Industry Partner Laboratory by taking IVD pregnancy tests from the same batch of production. The number of IVD pregnancy tests used was 54 compact tests selected by simple random sampling. The samples were grouped into 3 groups with 18 compact tests per group to represent the detectability of the population after storage at the specified temperature.

Each group was stored in an oven at 40°C, 50°C, and 60°C. The reasons for choosing high temperatures that extreme temperatures were needed to accelerate product quality deterioration in calculating accelerated shelf life. The determination of these three temperatures was based on the nature of antibodies that will undergo drastic denaturation in the range of 40-60°C until deactivation at 80°C. Sample storage was carried out for 35 days at each temperature. Packaging and sample position in the oven became controlled variables, so before conditioning the samples, checks had to be carried out. The primary packaging in the form of compact test cassettes was then covered with aluminum foil seals and secondary packaging in the form of boxes. The packaging must be ensured to remain intact and sealed without any leaks. The sample placement position in the oven was aligned and a maximum stacking of 3 compact tests.

## Immunochromatography Reader Calibration

The calibration was conducted to ensure that the Immunochromatography Reader (IC Reader) provided accurate measurements. The IC Reader calibration was carried out by measuring the color signal intensity of the calibrator 6 times repetitively. The color signal intensity on the test line calibrator was 19.0 mAbs, while on the control line calibrator was 208.7 mAbs. In this study, the calibration test was performed 3 times, specifically at every change in sample storage temperature observed. The criteria for the IC Reader calibration results were assessed using the standard deviation (SD) and percentage relative standard deviation (%RSD) to estimate the variability and consistency of the measurement results. The acceptance value of %RSD in the calibration is ≤2%. The SD describes how far the data in a sample deviates from its average, while % RSD represents the relative variation of the data compared to its average. A smaller SD value indicates that the data is closer to its mean, and a smaller %RSD indicates higher precision in the calibration. The results of the calibration presented in the following table.

**Table 1. Results of the First Calibration (40°C temperature).**

| Repetition of- | Calibrator Results (mAbs) | |
|---|---|---|
| | ***Control Line*** | ***Test Line*** |
| 1 | 206,6 | 18,6 |
| 2 | 206,8 | 18,7 |
| 3 | 206,7 | 18,7 |
| 4 | 206,7 | 18,7 |
| 5 | 206,7 | 18,8 |
| 6 | 206,8 | 18,8 |
| SD | 0,075 | 0,063 |
| %RSD | 0,036% | 0,338% |

The first calibration was carried out in February 2024, which was before observing the detectability of samples stored at 40°C. The results of the first calibration presented in Table 1. The SD value from the IC Reader calibration results on the control line was 0.075 and on the test line was 0.063. The %RSD value on the control line was 0.036% and on the test line was 0.338% so it can be concluded that the IC Reader has consistency and precision that can be trusted because it meets the %RSD requirement, which is ≤2%.

**Table 2. Results of the Second Calibration (50°C temperature)**

| Repetition of - | Calibrator Results (mAbs) | |
|---|---|---|
| | ***Control Line*** | ***Test Line*** |
| 1 | 206,8 | 18,8 |
| 2 | 206,7 | 18,7 |
| 3 | 206,7 | 18,7 |
| 4 | 206,9 | 18,8 |
| 5 | 206,9 | 18,8 |
| 6 | 206,8 | 18,6 |
| SD | 0,089 | 0,082 |
| %RSD | 0,043% | 0,436% |

The second calibration was carried out in April 2024, before observing the detectability of samples stored at 50°C. The results of the second calibration presented in Table 2. The SD value from the IC Reader calibration test results on the control line was 0.089 and on the test line was 0.082. The %RSD value on the control line was 0.043% and on the test line was 0.436% so it can be concluded that the IC Reader still has consistency and precision that can be trusted because it meets the %RSD requirement, which is ≤2%.

**Table 3. Results of the Third Calibration (60°C temperature)**

| Repetition of- | Calibrator Results (mAbs) | |
|---|---|---|
| | ***Control Line*** | ***Test Line*** |
| 1 | 206,8 | 18,5 |
| 2 | 206,9 | 18,6 |
| 3 | 206,9 | 18,6 |
| 4 | 207 | 18,7 |
| 5 | 206,8 | 18,7 |
| 6 | 206,8 | 18,7 |
| SD | 0,082 | 0,082 |
| %RSD | 0,039% | 0,438% |

The third calibration was carried out in May 2024, before observing the detectability of samples stored at 60°C. The results of the third calibration presented in Table 3. The SD value from the IC Reader calibration results on the control line was 0.082 and on the test line was 0.082. The %RSD value on the control line was 0.043% and on the test line was 0.436% so it can be concluded that the IC Reader still has consistency and precision that can be trusted because it meets the %RSD requirement, which is ≤2%.

## Determination of Critical Point

The Critical-Control Point/CCP is the value or limit at which a tool can still function properly or when the level of a material remains within safe limits. The determination of the critical point of the IVD pregnancy test at Industry Partner was carried out by approaching the concept of decreasing the detection signal value by 90% or having lost 10% of its initial ability. This critical limit indicates the minimum of Reaction Order value that must be maintained. The initial detectability value of the In Vitro Diagnostic test hCG product was 40.20 mAbs, which in the Arrhenius equation is known as A, the constant not affected by temperature. The calculation of the critical point loss was done by subtracting the initial detectability value of 40.20 mAbs by 10% so that the critical point of the IVD test in this observation was 36.18 mAbs. This critical point of 36.18 mAbs is the limit for shelf life calculations.

## Determination

**Table 4. Detectability observation results in the form of detection signals**

| Storage Day- | Detection Value (mAbs) | | |
|---|---|---|---|
| | **40**°C | **50**°C | **60**°C |
| 0 | 40,20 | 40,20 | 40,20 |
| 7 | 39,23 | 38,13 | 35,70 |
| 14 | 37,70 | 35,53 | 27,90 |
| 21 | 36,77 | 32,17 | 22,33 |
| 28 | 35,47 | 28,47 | 17,20 |
| 35 | 32,83 | 26,20 | 15,57 |
| Determination (R2) | 0,969 | 0,992 | 0,974 |
| Slope (k) | -0,200 | -0,418 | -0,752 |
| Intersep | 40,538 | 40,762 | 39,643 |

Table 4 shows the results of the IVD test product detectability observations. In the sample observations at each temperature, there was a decrease in detection values over time in storage. It can be seen that the higher the temperature, the faster the decrease in detection values of the IVD test. The decreasing detection values over storage time were caused by denaturation of antibody proteins in the test line portion. The test line contains specific antibodies that will later bind to hCG antigen complexes, colored particles, and hCG antibodies. When the temperature increases, the thermal energy received by the antibody molecules will also increase. This causes molecules within the antibodies to move faster, thus damaging non-covalent bonds such as hydrogen bonds, ionic bonds and hydrophobic interactions (Hamborg et al., 2020). The bonds of hCG antigen complexes, colored particles, and hCG antibodies will be damaged. Damage to the antibody protein bonds causes the IVD pregnancy test to not be able to exhibit color glow so it cannot be read by the IC Reader.

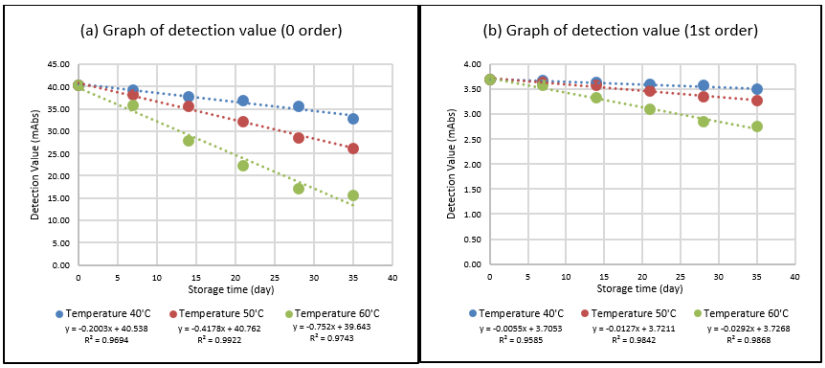


**Figure 2. (a) Graph of the relationship between decreasing detection values and storage time at each temperature for 0 order ; (b) Graph of the relationship between decreasing detection values and storage time at each temperature for 1st order.**

In kinetic reaction, quality deterioration in IVD test products may follow zero order and first order. Selection of the reaction order can be observed by making plots of quality deterioration data following zero order and first order. The determination of the reaction order is from the higher $R^2$ value of both linear regression equations obtained. If damage rate occurs constantly or linearly, it will follow a zero order reaction. However, if the damage rate occurs inconstantly, logarithmically or exponentially, it will follow a first order reaction. Determining the reaction order is a way to predict quality deterioration in shelf life calculations. In Figure 2, the decreasing detectability against storage time at each temperature can be seen the $R^2$ value of each equation. The $R^2$ value at 40°C and 50°C temperatures is higher in zero order than in first order, but at 60°C the $R^2$ zero order value is smaller than first order.

The slope value states the relationship between detection value and storage time. A negative slope value indicates a decrease in detection values in the IVD pregnancy test. This is because the IVD pregnancy test is highly sensitive to high temperatures. IVD pregnancy test exposed to high temperatures will experience damage, especially in the conjugate pad, test line and control line.

**Table 5. 1/T and ln k for Arrhenius Plot**

| Temperature | | 1/T | ln k | |
|---|---|---|---|---|
| °C | K | | 0 Order | 1st Order |
| 40 | 313 | 0,00319 | -1,6081 | -5,2086 |
| 50 | 323 | 0,00310 | -0,8727 | -4,3643 |
| 60 | 333 | 0,00300 | -0,2851 | -3,5332 |

In Figure 3, the results of the Arrhenius plot analysis show an $R^2$ value of 0.9998 for first order, while for zero order, the $R^2$ value is 0.9978. Because the determination value at first order is higher, the slope and intercept of first order will be used as the basis for determining the Arrhenius equation.

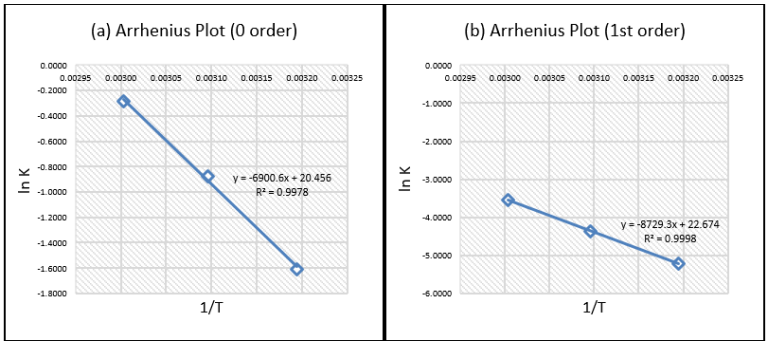


**Figure 3. (a) Arrhenius plot of ln k vs 1/T for zero order; (b) Arrhenius plot of ln k vs 1/T for first order**

## Determination of Shelf Life using the Arrhenius Equation

The result of the plot between ln k and 1/T will yield a regression equation, where the slope of that equation represents the value -Ea/R and the intercept represents the value ln A from the Arrhenius equation. The Arrhenius equation arranged as follows:

$$\ln k = -8729.3\ (1/T) + 22.674$$

The Arrhenius equation is then substituted with room temperature 30°C, which is assumed to be the normal storage temperature for IVD pregnancy tests. The substituted temperature is the absolute temperature in kelvin. The calculation will yield the value of k in the form of a natural log (ln).

$$\ln k = -8729.3\ (1/303) + 22.674$$

$$\ln k = -6.1356$$

$$k = 0.0022$$

The calculation of the shelf life of In Vitro Diagnostic pregnancy test products used storage conditions at room temperature, specifically 30°C/303K. Therefore, by substituting the temperature value in kelvin into the Arrhenius equation, the value of ln k = -6.6190 is obtained. Then exponentiated to get k, that is the quality degradation constant. The value of k = 0.0022, which means that there is a decrease in detectability of the IVD pregnancy test product 0.0022 mAbs per day. Thus, the total quality until expiration can be calculated by subtracting the initial detectability value of 40.20 mAbs with the critical poin value of 36.18 mAbs, resulting in a value of 4.02 mAbs. The estimated shelf life of the IVD pregnancy test product is calculated by dividing 4.02 mAbs by the daily quality decrease of 0.0022 mAbs, resulting in an estimated shelf life of 1857.25 days or 5 years. By obtaining a shelf life of 5 years, it can be concluded that the IVD pregnancy test in the sample batch can be stored for 5 years starting from the production period. Within 5 years the IVD pregnancy test can still maintain

its quality as claimed at 30°C room temperature storage.

## CONCLUSION

Based on the data analysis and discussion that have been explained, it can be concluded that the quality degradation constant value indicates a decrease in the detection signal of the In Vitro Diagnostic pregnancy test product 0.0022 mAbs per day. The calculation of the shelf life of the In Vitro Diagnostic pregnancy test product using the Arrhenius equation estimates the shelf life at room temperature of 30°C to be 1857.25 days or 5 years.

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