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Objectives: Accuracy evaluation of testing kit analyzing sperm ADN fragmentation in infertile men, Comparison improved testing kit (SSSperm testing kit) and Halosperm testing kit in the analyzing sperm ADN fragmentation.

Subjects and methods: A cross-section study was conducted on 300 semen samples from infertile men with sperm concentration ≥1 million/ml, Use Bland-altman, T-Test, Pearson tip to compare.

Results: The SSSperm testing kit has Coefficient of variation CV% = 2,26% < 5%; t_{tn}=0,97 < t_c, 2 methods have similar results DFI (r = 0,995; p<0,001). The difference between the results of two methods is very small and not statistically significant (p = 0,236 > 0,05).

Conclusion: The SSSperm testing kit analyzing sperm ADN fragmentation is qualified of quantitative tests, and the SSSperm testing kit analyzing sperm ADN fragmentation is equivalent to Halosperm testing kit.

Keywords: sperm adn fragmentation, dfi, infertile, improved, comparison.

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SUMMARY

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I. INTRODUCTION

Infertility is defined as the inability to achieve a clinical pregnancy after at least 12 months of regular unprotected intercourse (1). Recently, infertility tends to increase quickly and becomes a global health problem(2).Globally, there are estimated 15% of married couples affected by infertility, and male infertility accounts for 30-40% of these cases(3) (4). Male infertility can be caused by a lot of causes as testicle injuries,sperm problem, hormon problem...(5) , and one of the most important causes is sperm DNA fragmentation which affect sperm function completion and male reproductive health(6).

Today,in the world, there are a lot of methods of testing sperm DNA fragmentation such as Comet, Tunel, SCSA, SCD ... but these methods require modern equipment, complex techniques and high prices(7)(8).

In 2003, Fernandez and partners proposed Sperm Chromatin Dispersion test to determine sperm DNA fragmentation. This method is based on the principle that the sperms without DNA fragmentation will form large halos around its nucleus, while the sperms with DNA fragmentation will not produce halos or produce very small halos around its nucleus when it is denatured in acidic environment and remove the nuclear protein (9). Based on this principle, Fernandez and partners created the Halosperm testing kit in 2005, since then, the researches of sperm DNA fragmentation using the SCD method or Halosperm testing kit have been published, contributed significantly to the diagnosis and treatment of male infertility.

In Vietnam, some hospitals and research institutes have used the Halosperm kit to diagnose sperm DNA fragmentation, but due to complete importation, the cost of test is still high and not suitable for most patients'condition. So that, our research team has built and evaluated

the accuracy of an SSSperm testing kit to determine the degree of sperm DNA fragmentation by SCD method with the goal of completing the process and cutting the costs but still ensuring the quality of kit in assessment the degree of sperm DNA fragmentation of Vietnamese men. . However, at present, in Vietnam, there is no self-phase kit that can ensure the completeness as well as the accuracy of the test to determine the level of sperm DNA fragmentation. Therefore, we conducted this research with the aim of evaluating the equivalent of the SSSperm testing kit and the Halosperm testing kit using the Bland - Altman, T - test and Pearson test.

II. SUBJECTIVES AND RESEARCH METHOD

2.1 Subjectives

300 semen samples of male patients who were diagnosed with infertility at Hanoi Medical University Hospital, tested and assessed sperm DNA fragmentation at the Genetic counseling center, Hanoi Medical University hospital.

Selection criteria: Male patients aged from 18 years old, whose semen analysis has sperm density ≥ 1 million / ml and agree to participate in the research.

Exclusion criteria: Male patients who do not meet the above criteria, have genital cancer, are infected with HIV, syphilis, gonorrhea, have acute disease, mental illness and patients who disagree to participate in the research.

III. RESEARCH METHOD

3.1 Sample size

To complete the procedure, determine the accuracy, we use the formula to calculate sample size for a discriptive research according to S.K. Lwanga and Lemeshow's formula[5]:

$$n = Z^2_{1-\alpha/2}\frac{1-p}{\epsilon^2 p}$$

In which: 1- α/2 = 0.95; ε = 0.10; p = 95% (accuracy of reference procedure), n = number of required experiments, calculate at 21, we double and round to 50.

To compare SSSperm testing kit with Halosperm testing kit, we use formula to calculate sample size:

$$n = Z^2_{(1-\frac{\alpha}{2})}x\ \frac{p(1-p)}{(\varepsilon p)^2}$$

$Z_{(1-\alpha/2)}$: reliability coefficient (with 95% confidence ,Z= 1,96).

p: According to Duran E.H's research in 2002, the rate of high sperm DNA fragmentation >30% was p= 25% [7].

ε: we select 0,2.

n = $1{,}96^2$ x 0,25 x (1 – 0,25) : $(0{,}2 \times 0{,}25)^2$ = 147, rounded to 150.

We use a sample size of 300 to increase the accuracy.
So we used a sample size of 300.

3.2 Research design

A cross-sectional study.

3.3. Method of making templates

The test (using SSSperm testing kit) is improved based on Fernandez's SCD procedure (2003) [3], using Halosperm kit of Halotech as follows:

Step 1. Preparation of agar: Place agarose eppendorf tube into the float and melt using a water bath at 95 - 100 ° C for 5 minutes or in microwave for 3 minutes, until it is completely melted . Dilute semen samples with PBS solution so that the concentration of sperms is approximately <15 million / ml . Keep the agarose tube at 37 ° C for 5 minutes until the temperature of the eppendorf containing agar and the temperature of the incubator is balanced.

Step 2. Preparation of cell suspension : add 25 µl of semen to an agarose tube and mix well with a pipette. Keep the tube at 37 ° C and quickly take the next step, avoid agarose solidifying. Drip a drop of 25µl of cell suspension on a circular position on the microscope slide, cover the microscope slide, gently press, to prevent air bubbles from appearing. The microscope slide

must be held horizontal throughout the entire process. Place the template in a refrigerator at 4 ° C, for 10 minutes, to allow agarose to solidify.

Step 3. After the cell suspension has solidified, remove the template from the refrigerator and remove the microscope slide by gently sliding away.

Preparation denatured solution and denaturation of sperm DNA: take 80 µl of denaturing solution into a tube containing 10ml of distilled water, shake well and we get the necessary denaturation solution. Place the template in the tray containing denaturing solution for 7 minutes.

Step 4. Cell lysis: take the template from denaturing solution and place in a tray containing 10 ml of lysis solution for 5 minutes.

Step 5. Wash the lysis solution: After finishing the lysis step, place the template in the tray containing distilled water for 5 minutes to wash the lysis solution.

Step 6. Dehydration: Dehydrate by adding the template to the alcohol solution for 6 minutes, then allow to air-dry.

Step 7. Dye the template: place the template horizontally, drip Giemsa solution 5 - 30% on the surface of the template, leave at room temperature for 10 minutes and then wash with water from the tap, avoid excessively washing which lightens the halo color.

IV. DATA PROCESSING

a) Evaluate the results

Observe the microscope slide under an optical microscope, and count at least 500 sperms on the template to determine the degree of sperm DNA fragmentation. Sperm DNA fragmentation was determined by sperm halo according to Fernandez et al.

The rate of DNA fragmentation (DFI - DNA Fragmentation Index) is determined by the following formula:

$$\frac{sperms\ \ having\ small\ halo+sperms\ \ having\ no\ halo+degenerative\ sperms}{Total\ counted\ sperms} \times 100\%$$

b) Data analysis

*To evaluate the accuracy of SSSperm testing kit

Evaluate accuracy of the testing kit through two indicators: trueness and precision [6]:

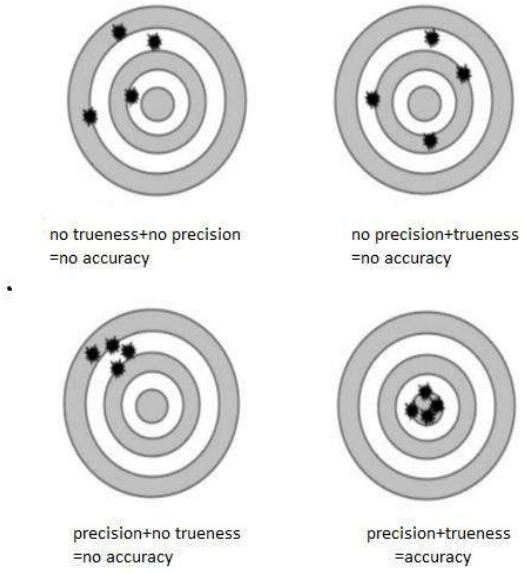


Figure 1: Illustration of accuracy [6]

Precision: Precision is the degree of variation of independent test results around the mean. Precision is a qualitative concept and is expressed quantitatively by standard deviation or coefficient of variation. The lower the precision is, the larger the standard deviation or coefficient of variation is.

$$SD = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}}$$

$$RSD\% = CV\% = \frac{SD}{\bar{x}} \times 100$$

In which:
SD: standard deviation
n : number of experiments
xi: Calculated value of the "i" experiment
$\overline{X}$: The average value of the experiments
RSD%: Relative standard deviation
CV%: Coefficient of variation

The precision can be classified into three following cases:

-Repeatability

Express the degree of accuracy or repeatability, the degree of variation among experiment results which are done in:
+ Same laboratory.
+ Same homogeneous sample.
+ The same inspector.
+ Same period of time.

Repeatability is determined by the method: on a patient's semen sample, use a improved kit (SSSperm kit) to determine the degree of sperm DNA fragmentation , repeat 10 times. Calculate SD standard deviation and CV% coefficient of variation with CV requirement ≤ 5%.

- Intermediate precision:

Express the accuracy of the method according to variables of laboratory :
+ For several days.
+ With different inspectors.
+ With different tools.

- Reproducibility
Express the accuracy of many laboratories conducting studies on the same homogeneous sample. Similar to repeatability provided that:

+ Change laboratory
+ Change method.

Trueness: indicates the degree of proximity between the average of the experimental results and the real value or accepted value is truly μ.

Determining the accuracy by the method : on a patient 's semen sample being determined the degree of sperm DNA fragmentation by Halos-perm kit, conduct experiment by using SSSperm testing kit, repeat 10 times, calculate the average value and standard deviation, from which the standard t_{tn} is calculated using the following formula, and then compared with Halosperm kit

$$t_{tn} = \frac{|\mu - \bar{x}|}{\sqrt{\frac{S^2}{n}}}$$

In which: t_{tn} : experimental t value

$t_c(\alpha,k)$: t value taken from table with statistical significant 0.5%

μ : real value or accepted value (reference)

$\bar{x}$: mean of experimental method

S^2 : variance of experimental method

n : number of experimental times

*To compare SSSperm testing kit with Halosperm testing kit:
Investigating the difference between the two methods is based on: Pearson correlation analysis, T - test and Bland-Altman plot using Epidata and SPSS.20 software.

V. ETHICAL RESEARCH

All the patients' information is kept confidential and only analyzed for fertility counseling for the patients and for this study, not for any other purposes.

VI. RESULTS AND DISCUSSION

*Accuracy evaluation of testing kit analyzing sperm ADN fragmentation in infertile men:

On a semen sample that had been identified DFI by using Halosperm kit, we used an improved kit(SSSperm kit) to determine the degree of sperm DNA fragmentation, repeated10 times. The results are in the following table:

Table 1: Results of test determining the accuracy of the SSSperm kit

Time of Experiment	DFI (%)
1st	15,4
2st	15,0
3st	14,4
4st	14,2
5st	15,2
6st	15,2
7st	15,2
8st	15,0
9st	14,6
10st	15,0
Proof (made of Halo kit)	14,8

6.1: Precision

Because of conducting in the laboratory, we calculate the precision through the repeatability. From the above result table we get:

Table 2: Results of precision evaluation

The mean of DFI (%)	14,92
SD	0,391
CV%	2,62%

In experiments, especially in quantitative tests, there are many errors that affect the test, lead to inaccuracy in the results. Therefore, to control these confounding factors, it is necessary to use the concept of precision. The precision describing the results only depends on the random errors and do not relate to the actual results of the sample. The lower the precision is, the larger the standard deviation or coefficient of variation is, otherwise, the greater the precision is, the smaller the coefficient of variation is [6]. In this study, our improved kit has repeatability with coefficient of variation CV% = 2.62% .So, the coefficient of variation has a value not exceeding 5% according to the Vietnam Standards [6], this indicate that repeatability of the procedure meets the requirements of the analysis. Thus, when there are effects of random error factors, for the same sample, the degree of sperm DNA fragmentation determined under different conditions has errors within the acceptable range.

Compared with the commercial Halosperm kit of Fernandez which has an actual coefficient of variation of 5.3% [7]; higher than the SSSperm kit. This proves that the SSSperm kit meets the standards of a testing kit.

6.2: Trueness

Trueness indicates the degree of proximity between the average values of the experiment results and the real values or accepted values are true

With experimental testing the trueness, we calculate t_{tn}= 0,97; Besides, through searching tables, t_c = 2,262 [6]. Thus $t_{tn} < t_c$. This means that the sperm DNA fragmentation index determined by the SSSperm kit has the same results as by the commercial Halosperm kit. The process achieves the accuracy as requirements of an analysis.

Thus, the precision and the trueness of the SSSperm kit completely meet the requirements of a testing kit according to Vietnamese Standards. This is the first step of the project.

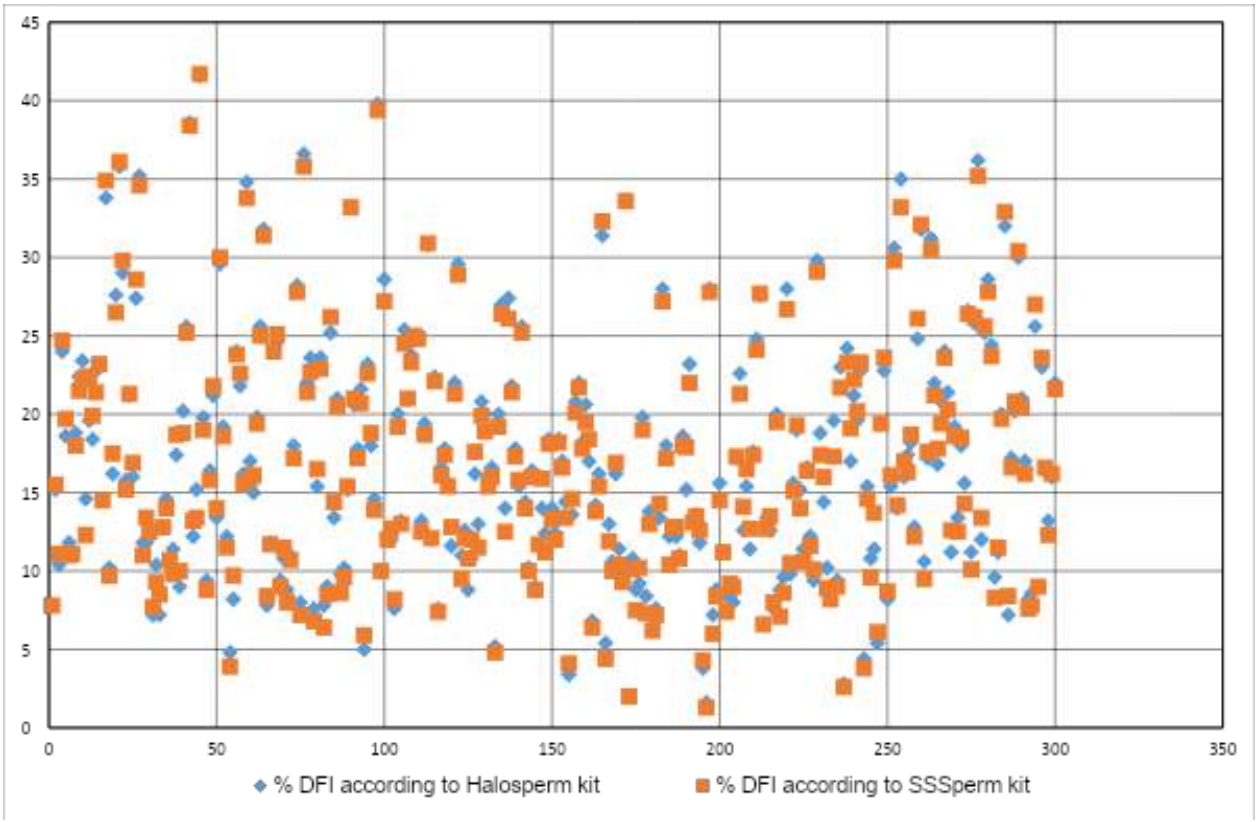
- *Compare SSSperm kit with halosperm kit*

We have developed an improved procedure for determining the level of sperm DNA fragmentation that is different from the Halosperm testi kit at the following key points:

Table 3: Improvements in techniques for testing sperm DNA fragmentation

	Fernandez et al, 2003 [6]	SSSperm testing kit
Denaturing solution	Denaturing solution of kit	HCl 0.29%
Lysis solution	Lysis solution 1: 0,4 M Tris-HCl; 0,8 M DTT; 50 mM EDTA; 1% SDS, pH 7,5. Lysis solution 2: 0,4 M Tris -HCl; 2 M NaCl; 1% SDS, pH 7,5	0,2 M Tris ; 0,1M DTT 2 M NaCl ; 1% Triton, pH 7,5
Dehydration	3 steps with alcohol 70%, 90% and 100%	1 step with alcohol 100%
Dyes	Wright	Giemsa

After completing the SSSperm testing kit, we took 300 semen samples to make templates to assess the degree of sperm DNA fragmentation by 2 methods with the Halosperm testing kit and SSSperm testing kit. Our results are shown in following chart:



Comment:

Value of the sperm DNA fragmentation index (DFI) measured by the Halosperm commercial kit and the SSSperm testing kit are almost similar.

To compare two kit more accurately, we Pearson test, T - test and build Bland - Altm plot.

Table 4: Table of testing correlation coefficient between two methods

N		300
Pearson correlation coefficient		0,995
p		< 0,001
confidence interval 95 %	Upper limit	0,996
	Lower limit	0,994

Comment:

Pearson test showed a strong and significant correlation between sperm DNA fragmentation index measured by two methods : using SSSperm testing kit and commercial Halosperm kit with r = 0.995; p <0.001.

Table 5: T-test table

t	p	The mean of the difference	confidence interval 95%	
			Lower limit	Upper limit
1,187	0,236	-0,010	-0,003	0,011

Comment:

Results of assessing the level of sperm DNA fragmentation by using SSSperm kit and by using commercial Halosperm kit do not have statistically significant differences with 95% confidence level (p = 0.236> 0 , 05).

Using Bland - Altman plot which is used to quantify the compatibility between two different measurements or to compare a new test with a standard recognized test.

From the above tests, we have built a Bland Altman plot showing the compatibility between measurement results of two methods:

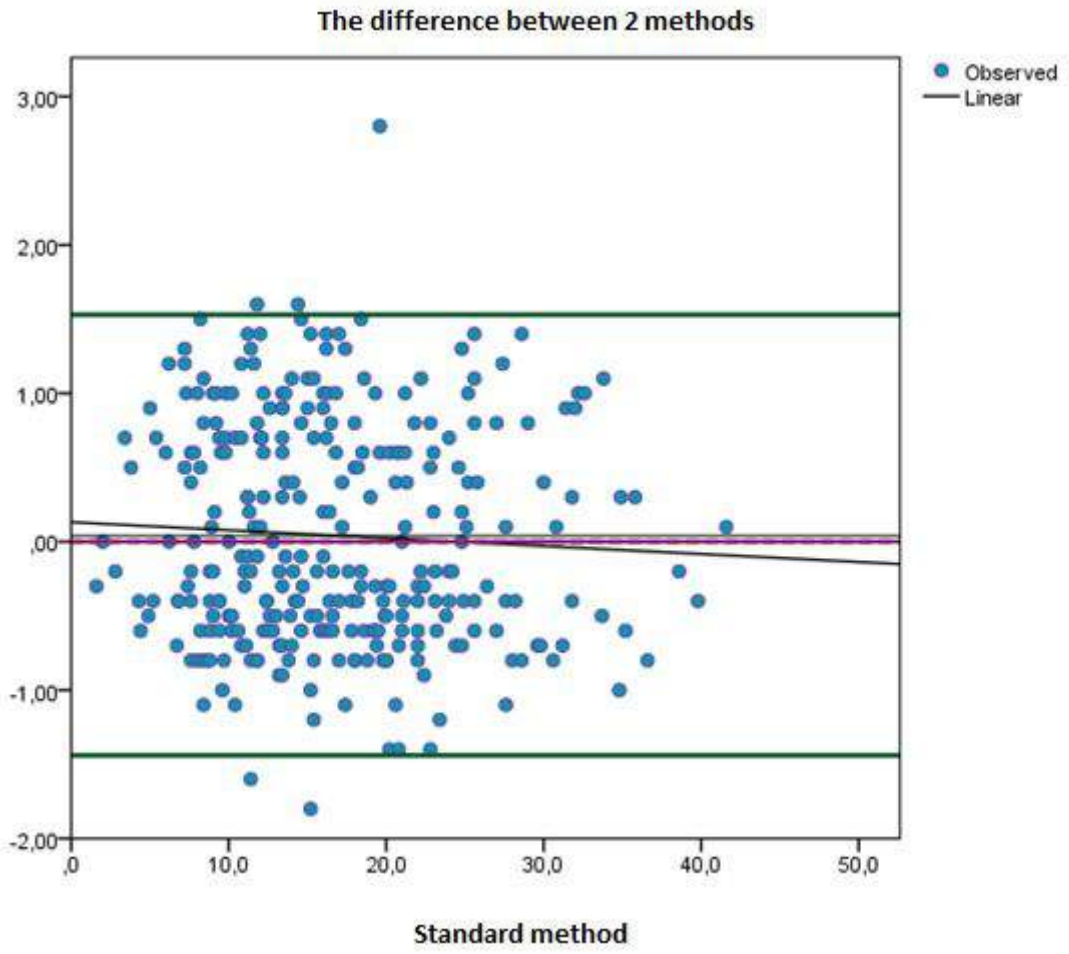


Figure 2: Bland Altman plot showing the compatibility of the two measurement methods

Comment:

The difference of mean between the two methods is very small (0.042). Most cases have errors within the limit of ± 1.96 standard deviations. Therefore, the two methods : testing by SSSperm kit and commercial Halosperm kit have the same value in determining the degree of sperm DNA fragmentation.

VII. DISCUSSION

Sperm with fragmented DNA inable to produce the halo of dispersed DNA loops while the normal sperms succeed in producing the halo after treatment with denaturing agent and removing nuclear protein.Basing on this principle, we created an improved test (SSSperm kit) to determine sperm DNA fragmentation.

What are the advantages of the SSSperm kit and the differences between the improved test and other existing tests ?

The improved test is a quantitive test . Unlike semiquantive tests as COMET, TUNEL....which determine sperm DNA fragmentation by determining color and flourscence intensity, the improved test determines sperm DNA fragmentation by measuring percentage of sperms with nondispersed (have no halo or small halos) or dispersed DNA loops (have large halos), which can be looked with naked eyes.

Halosperm testing kit which is also based on principle that the sperms with fragmented DNA fail to produce halos while normal sperms produce large halos was published by Fernandez et al in 2003 .There have been some researches conducted to evaluate the value of this kit(9) . The results obtained from these reseaches indicated

that this testing kit meets the accuracy requirement to determine sperm DNA fragmentation and it has been used widely in diagnosing male infertility especially in Viet Nam .

However the price of this kit is still high which is not suitable for many of VietNamese . Therefore , we created the improved testing kit(SSSperm testing kit) which is simpler and cheaper than Halosperm testing kit but still ensures the quality of the new kit. When we use Pearson test, T-test and Bland –Altman plot to compare the SSSperm testing kit with Halosperm testing kit, the results indicated that there were sigfinicant correlations between the two kits ($r^{=}0.995, p<0.001$), the mean of difference was -0.01, $p^{=}0.236>0.05$, the difference was not statically significant.

In conclusion, the improved test is accurate, fast, inexpensive and simple . Therefore , the SSSperm testing kit should be used as a routine kit in Viet Nam to determine sperm DNA fragmentation for infertile men.

VIII. CONCLUSION

The SSSperm testing kit has the required accuracy of a quantitative testing kit (with CV% = 2.62% <5% and ttn = 0.97 <tc).

Result obtained from improved process is equivalent to the commercial Halosperm kit. Differences in the results obtained from the two methods are not statistically significant, and are completely random.

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