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Verifying cosmetics products made with caviar extract using the Syber Green Quantitative PCR technique

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ABSTRACT

The verification of biomaterial used in cosmetic products needs to be tested through some techniques such as hybridization, DNA-binding protein, fluorescence dye, and quantitative PCR, all based on identifying protein or DNA remaining in the product. In this research, a real-time PCR method was used to evaluate the remaining DNA of caviar extract, which is based on the use of Syber Green buffer. At first, DNA extraction was performed from the fin tissue of the Caspian Sea sturgeons, including Siberian and Sterlet sturgeons, as a model for fragment amplification. So, a small cytochrome oxidase I gene fragment was amplified using the conventional PCR. To verify the presence of DNA in caviar extract using the real-time PCR method, a standard curve was drawn using the DNA of the fin tissue of Siberian sturgeon. The primer specificity for sturgeons was tested using negative control DNA like human, yeast, and bacteria. Results showed that all seven sturgeons amplified a fragment of *CoI* gene with specific sturgeon primers. A standard curve was drawn with a linear relationship between threshold cycle values, and a coefficient higher than 98% was obtained with the real-time PCR method. According to the standard curve, unknown samples of caviar cream DNA concentration ranges were 2 ng - 400 pg. None of the irrelevant genomic DNA (human, yeast, and bacteria) was indistinguishable from the response of the negative control when analyzed with the *COI* sturgeon's specific primer. In this research, real-time PCR could detect picogram and nanogram levels of residual DNA in cosmetic products, and the verification of caviar cream was approved for making it with caviar extract using this sensitive technique.

Keywords: Cosmetics, Caviar extract, Real time PCR, *COI*, Barcoding.

INTRODUCTION

One of the ways to detect precisely the use of caviar extract compounds in cosmetic products is through the PCR-based molecular method. This technique utilizes the remaining DNA in the cosmetic product for specific band amplification. The amplified PCR fragments are then sent for sequencing. This method determines the possibility of sturgeon DNA presence in the product. Also, the species of sturgeon applied for caviar oil will be identified. However, this method is time consuming and, in some cases, extracting, amplifying, and detecting low concentrations of DNA in cosmetic product is very hard or cannot to be determined precisely. Jamshidi & Hassanzadeh Saber (2021) extracted DNA of sturgeon caviar in cosmetic product (about 5-20 ng μL^{-1}), and PCR-sequencing detected species. In the drug production industry, it is pretty standard to use *Escherichia coli* bacteria to produce protein drugs and recombinant proteins, since they grow quickly and cheaply in general culture media and has a well-known genetic system (Banexy1999). However, the DNA of the bacteria must be extracted and purified from the products (Hoffman 1990; Rathore *et al.* 2003; Shukla *et al.* 2008), since it may affect the genetic system of consumers (Walsh 2002). The American Food and Drug Administration (FDA) has declared the permissible amount of DNA remaining in biological products as 100 picograms per dose of the product, and according to The World Health Organization and European Union, it has been declared 10 nanograms per dose of the product (Eldering *et al.* 2004). DNA barcoding, particularly using the *COI* (cytochrome c oxidase subunit I)

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gene in mitochondria DNA, is a molecular method for identifying species based on a short, standardized DNA sequence (Ward *et al.* 2005; Smith *et al.* 2005; Hajibabaei *et al.* 2006; Chan *et al.* 2014; Hu *et al.* 2023). In animals, a fragment of the mitochondrial *COI* gene is commonly used as a barcode. This method allows for rapid and accurate species identification by comparing the obtained DNA sequence to a reference library of known species. DNA barcoding, specifically using the *COI* gene, is a common method for identifying many fishes, since evolution of this gene is slow in many groups of animals (Ivanova *et al.* 2007; Hu *et al.* 2023; Filonzi *et al.* 2024) and sturgeon species and verifying the authenticity of caviar. It is particularly useful in cases where morphological identification is difficult or impossible, such as processed caviar.

In molecular biological science, some methods used to determine the residual DNA content of biopharmaceuticals include hybridization, DNA-binding protein, q-PCR[1], and fluorescence dye. All of these methods are semi-quantitative or quantitative assays (Kung *et al.* 1990; Singer 1997; Ji *et al.* 2002; Desjardins & Conklin 2010; Kang 2011; Wang *et al.* 2013; Rathore 2013). In the pharmaceutical industry, the real time PCR method is used to amplify DNA and determine its quantity simultaneously to identify the specific sequence of DNA and its amount (Kubista *et al.* 2006). This method has also been used to identify Chinese hamster ovary DNA in medicinal products (Nissom 2007). A diagnostic of protein caviar in a cosmetic product would involve identifying and quantifying the protein components derived from caviar and assessing their impact on the product's efficacy and stability. This can be achieved through various analytical techniques, including: electrophoresis, chromatography, spectrophotometry, and immunological assays (Pratiwi *et al.* 2023; Momeni *et al.* 2024). Given the time-consuming, expensive, and low sensitivity of other methods, this study aimed to develop a method for detecting the residual sturgeon DNA based on Syber Green Quantitative Real-Time PCR. This method is cost-effective and more convenient for the verification of cosmetic products made with caviar extract or products. It will contribute to a diagnostic method based on SYBR Green real-time PCR. Also, this method allows highly efficient, rapid, and accurate differentiation of sturgeon from other fish and animals based on the *CoI* gene sequence, which makes it possible to detect a few about-tenth picogram levels of the caviar extract DNA. Besides, in terms of application in detecting DNA residues in cosmetics, this method (Real time PCR) has been carried out for the first time in Iran for caviar verification and have a novelty in diagnostic tests in our country.

MATERIALS AND METHODS

Sample preparation, DNA extraction from fin tissue, and cosmetic products (Caviar cream)

A total of seven tissue samples belonging to five pure species of the Caspian Sea sturgeon and two species belonging to farmed sturgeons (*Acipenser ruthenus* and *A. baerii*) were analyzed in this study (Table 1) as control positive of Acipenseridae. Ten milligrams of the fin tissue powdered by liquid nitrogen were used for DNA extraction via QIA gene extraction protocols (Qiagen Blood and Tissue DNA Extraction Kit). An aliquot of 100 µL of three Caviar cream was applied for DNA extraction via QIA gene kit. The quantity of extracted DNA was measured by Nanodrop set (Thermo Fisher, Nanodrop-1000).

Primer selection for sturgeon DNA tracing and Conventional PCR amplification

ACoI Primer pairs for amplifying sturgeon's *CoI* gene were selected from the Waraniak *et al.* (2017) study. Primer-BLAST in NCBI was employed to confirm the theoretical specificity of the selected primers, while the final specificity was ensured through a practical PCR amplification against target species (sturgeons) and non-target species. These primer pairs had been designed for amplifying the Mini-Barcoding fragment of the *CoI* gene in *A. fulvescens,* but have very few mismatches with other species of Acipenseridae theoretically and might be capable of amplifying the DNA of other species of sturgeon. To confirm the correctness of the amplification bands in the real-time PCR mini-barcoding method, at first, conventional PCR was done. All PCR reactions were performed in a total volume of 10 µL, consisting of 5 µL Red Master Mix buffer of Ampliqon, 0.2 µl each forward and reverse primers, and 100 ng template extracted DNA (tissue DNA). All amplifications for *ACoI* were performed on Eppendorf PCR System set as follows: 5 min at 94 °C, 35 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, followed by a 5-min extension at 72 °C. Products were checked by 1.8% agarose gel electrophoresis.

[1] Quantitative PCR

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Standard curve (linearity) drawing for real-time PCR and checking primer's specificity in sturgeons

The linearity of the analytical procedure was determined by the seven-fold serial dilutions (10 ng, 2 ng, 400 pg, 80 pg, 16 pg, 3.2 pg and 0.064 DNA) of genomic DNA. The amplification was performed with BioRad CFX96 real-time PCR system using the following conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 56 °C for 10 s, and 72 for 20 s. Fluorescence signals were measured at the end of each cycle on the SYBR green channel. Two PCRs were performed in parallel. For the standard curve, serial dilutions of reference DNA (*A. baerii*) were prepared in water, and a fixed volume (20 µL) of each dilution was tested as described above. At the end of the reaction, the standard curve was generated based on plotting the logarithm of DNA concentration (horizontal axis). Five Caspian sturgeon species as well as Sterlet, and Siberian sturgeon were tested for amplification by *CoI* primers. Also, a test was done for targets (five Caspian sturgeon species, Sterlet and Siberian sturgeon) and non-target species.

Real-time PCR assay, thermal cycling and verification of cosmetics products (Caviar cream)

Quantitative Real-time PCR was conducted in a final volume of 20 µL containing 10 µL of 2X SYBER Green (Ampliqon), 100 nM of each primer to determine the amount of sturgeon DNA in cosmetic material using specific primers for the sturgeon. Real-time program and conditions were the same as those for standard curve amplification. Melt curve analysis was performed by cooling amplification products at 55 °C for 90 s and then heating from 55 to 95 °C with a ramping rate of 0.5 °C/5 s. For each sample, two parallels were performed. The Q-PCR reaction mix contained a final concentration of 300 nM for each primer. A sample with no DNA was used as the negative control.

RESULTS AND DISCUSSION

The concentration of tissue-extracted DNA samples of five Caspian Sea sturgeons, Sterlet, and Siberian sturgeon was between 150-370 ng μL^{-1}, but the DNA concentration of caviar samples was 5-20 ng μL^{-1} measured by Nanodrop. Primer-BLAST in NCBI showed specific amplification of sturgeon's mitochondrial DNA. Also, a practical PCR amplification for each of the five Caspian Sea sturgeons, Sterlet, and Siberian sturgeons confirmed the successful amplification of the Mini-Barcoding fragment in these species (Fig. 1) with tissue DNA of species. Additionally, a linear relationship exists between the threshold cycle (*ct*) value and the DNA concentration measured by the Mini-Barcoding quantitative real-time PCR (Fig. 2). DNA samples of cosmetic material (caviar cream) showed by × = unknown samples and have a concentration range between 2 ng to 400 pg. The residual DNA of sturgeons was assayed in caviar cream material. The desired amplified fragment is denatured at a temperature of 82.5 ºC for sturgeons Mini-barcoding primer (Fig. 3), showing non-specific for irrelevant genomic DNA and one product amplification (melt curve in Fig. 4). The B line indicates the non-binding of primers (primer dimer) in negative control (Fig. 3). The DNA extracted from human cells (*Homo sapiens*), yeast (*Saccharomyces cerevisiae*), and bacteria (*Escherichia coli*) were indistinguishable from the response of the background (negative control, NTC) when analyzed with the *ACoI* primer.

Specificity test for sturgeon sample showed all seven sturgeon genomes (Caspian Sea sturgeons and *A. baerii,* and *A. ruthenus*) amplified a fragment (~135 bp) but irrelevant genomic DNA, were indistinguishable from the response of the background (NTC) when analyzed with Mini-Barcoding primers (Fig. 4). Amplifications curve of consecutive DNA dilutions of sturgeon and DNA dilutions of cosmetic samples from cosmetic products (caviar cream) showing Mini-Barcoding primers capable of detection of a few residual DNA of sturgeons in processed food at the tenth picogram level of Caviar extract DNA (Fig. 5).

In the cosmetics industry, it is common to use caviar extract or caviar oil to produce caviar cream, but verifying the authenticity of using caviar extract in products is not easy, since during processing for caviar extract production, the remaining DNA of sturgeon in products is not significant for routine molecular detection of species detection and sturgeons barcoding in cosmetic products need a few about tenth picogram level of DNA in Caviar extract and conventional method like PCR amplification and gel electrophoresis could only show band in nanogram level of DNA in samples. Therefore, in this study, a highly sensitive and accurate real-time quantitative PCR (q-PCR) method was used to detect the residual DNA of sturgeon in caviar extract based on a specific sturgeon primer, which initially chosen by Waraniak *et al.* (2017) for sturgeon eDNA detection in nature. Furthermore, real-time PCR, as used in this study, is capable of detecting DNA of sturgeon in the cosmetic products, such as caviar cream, with suitable sensitivity and cost-effectiveness.

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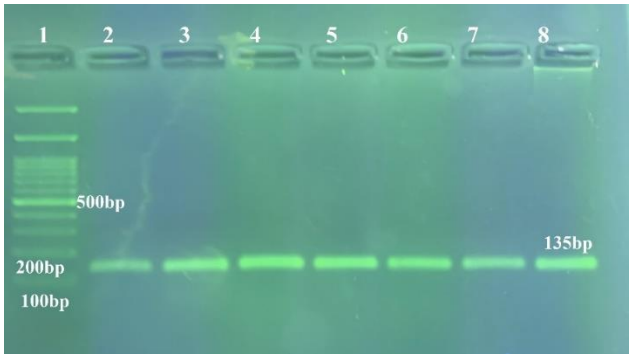

Fig. 1. Successful amplification of Mini-Barcoding sturgeon's primer bands in Caspian Sea sturgeons 1: DNA 100bp ladder Sinaclone; 2: *Acipenser persicus* (Persian sturgeon); 3: *Acipenser stellatus* (Starry sturgeon); 4: *Acipenser gueldenstaedtii* (Russian sturgeon); 5*: Acipenser nudiventris* (ship sturgeon*)*; 6: *Huso huso* (Beluga)*;* 7: *Acipenser ruthenus* (Sterlet) and 8: Siberian sturgeon (*Acipenser baerii*) with sturgeon's tissue DNA.

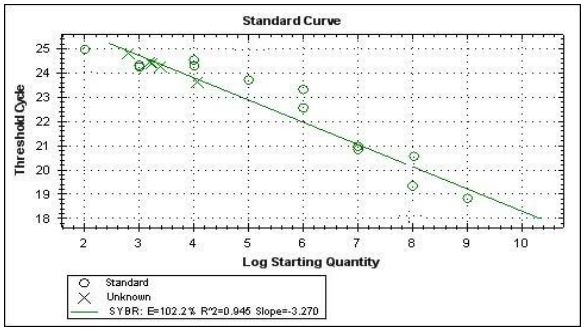

Fig. 2. Standard curves of genomic DNA of *A. baerii* (two repeat tests for each dilution) with consecutive dilutions from 10 ng to 0.064 pg (it was diluted in a ratio of 1 to 5) showing a linear relationship between threshold cycle (CT) values and the DNA concentration measured by the *ACoI* quantitative real-time PCR amplified in BioRad CFX96 real-time PCR system. A correlation coefficient higher than 98% was obtained for *ACOI* primer in *A. baerii*. The unknown sample concentrations (caviar cream) range, shown by × symbols, ranged from 2 ng to 400 pg according to the Fig.

The results of this study showed that only a few picograms of residual sturgeon DNA in cosmetic products could amplified a specific *ct* value for verifying the authenticity of using caviar extract in products. This method is conveniently used for the verification of cosmetic products made with caviar extract and it will contribute to a diagnostic method based on SYBR Green real-time PCR for specific barcoding of sturgeons. In 2006, the hybridization method was applied to identify residual host cell DNA in DNA vaccine products as a recombinant drug. However, its sensitivity was insufficient, and it was time-consuming and expensive (Wang *et al.* 2006).

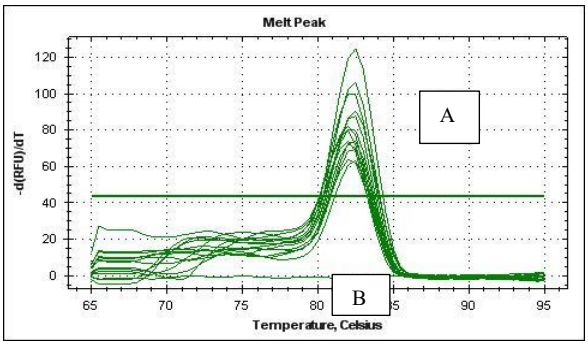

Fig. 3. Melting curve based on temperature (horizontal axis) and fluorescent signal derivative (vertical axis) received from BioRad CFX96 Real-Time PCR system amplified in *A. baerii* in different DNA dilutions. It shows that the desired amplified fragment is denatured at a temperature of 82.5 ºC. B indicates the non-binding of primers (primer dimer) in the negative control.

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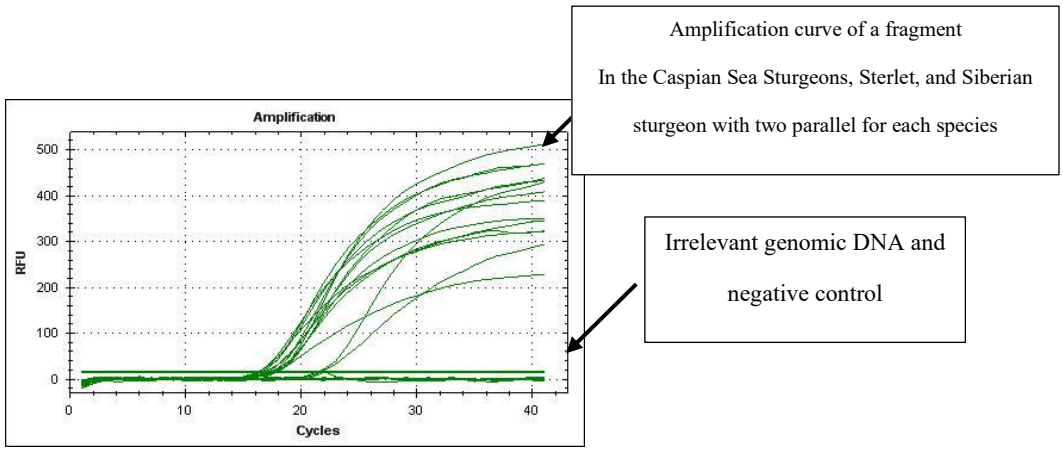
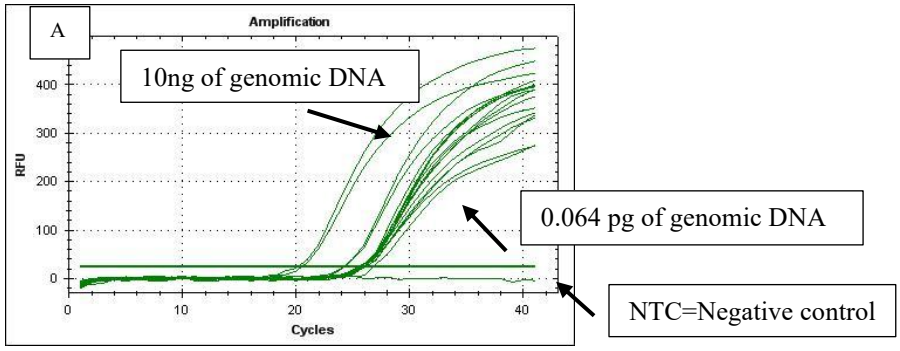


Fig. 4. Specificity test for sturgeon samples, only the sturgeon genomes have an amplification curve. All seven sturgeon genomes (Caspian Sea Sturgeons, *A. baerii,* and *A.ruthenus*) amplified a fragment, but irrelevant genomic DNA, human (*Homo sapiens*), and a bacteria (*Escherichia coli*) were indistinguishable from the response of the background (NTC) when analyzed with the *ACOI* primer. Two PCRs were performed in parallel for each species.

In 2010, Lee *et al.* compared the hybridization method compared with real-time method for detecting Reovirus Type 3 in Mammalian Cell Culture. They concluded that real-time qPCR is 30 times higher in sensitivity and accuracy. It has been shown that the most laboratories prefer quantitative PCR (qPCR) or digital PCR (d PCR) methods due to the low detection limits of these assays (Hussain 2015; Fan *et al.* 2024). There are much more specific methods based on TaqMan probes and quenchers, such as FAM[2], TET[3], and TAMRA[4] which offer significantly higher sensitivity in diagnosis. In addition to barcoding sturgeon, these methods also detect the species of fish; however, the cost of using them is prohibitive. Despite the advantages of the SYBR Green method, such as its affordability and ease of use; one of its defects is the production of primer-dimer, which can be minimized by optimizing the concentration of primers, the concentration of Mg cations, and adjusting the annealing time and temperature (Lovatt 2002). In 2025, Dodd *et al.* developed a robust DNA barcode method to detect foreign sugar adulteration in honey. In their research, the analysis of sugar composition was conducted using HPLC, alongside qPCR DNA tests, to supplement the method with additional data. Wajahat *et al.* (2022) confirmed the potential of DNA-based real-time PCR for the adulteration of camel milk from goat and cow milk and molecular species barcoding. Among quantitative or semi-quantitative methods, quantitative assay methods were used to determine the residual DNA content of products, including hybridization, DNA-binding protein, and q-PCR; the last one was used to amplify DNA and determine its quantity at the same time in order to identify the specific sequence of DNA and its amount for Barcoding (Kubista *et al.* 2006). Mamnon *et al.* (2015) studied some interferon samples for residual DNA assays and revealed that the amount of DNA impurities was about 0.02 pg per product dose.



[2] 6-carboxyfluorescein, acronym
[3] tetrachlorofluorescein, acronym
[4] tetramethylrhodamine, acronym

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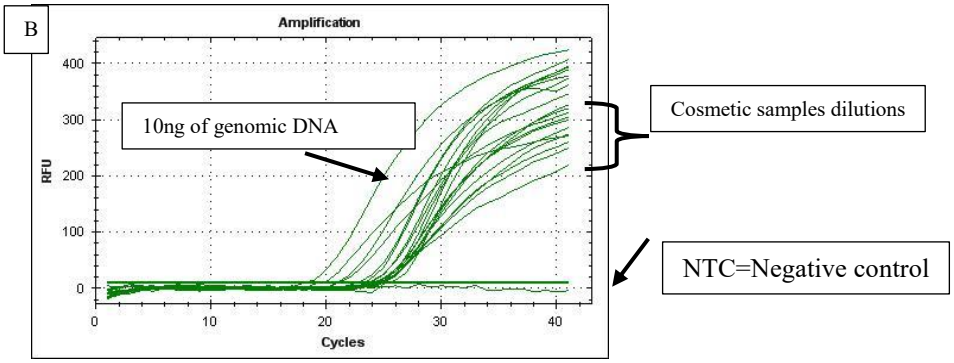

Fig. 5. Produced threshold cycle (*ct*) amplifications curve of consecutive DNA dilutions of sturgeon (Graph A and B) and DNA dilutions of cosmetic samples (500 pg,16.5 pg and 5 pg) from cosmetic products (Caviar extract) (Graph B); Graph A and B show generated amplification curve of Consecutive sturgeon DNA dilutions from 10ng to 0/064pg (it was diluted in a ratio of 1 to 5) and Graph B compares generated amplification curve of consecutive DNA dilutions of sturgeon with DNA cosmetic samples dilutions.

They found that real-time PCR can be used as a functional and accurate technique in manufacturing centers for detection of residual host cell DNA in interferon and other recombinant pharmaceutical products. The conventional PCR method is a simple yet effective method for identifying species, provided the amount of DNA is medium to high, and the target fragments of the *COI* gene should be sequenced for accurate species identifications (Jamshidi & Hassanzadeh Saber 2021). Mugue *et al.* (2008) identified different species of the Acipenserid family according to different sizes of amplified fragments of the Mitochondrial control region on agarose gel electrophoresis. However, this method requires at least a nanograms amount of DNA and a picogram level of DNA in processed products. In most cases, any bands cannot be amplified.

CONCLUSION

In this research, the Caspian Sea sturgeons, Sterlet, and Siberian sturgeons amplified a fragment with a specific sturgeon primer from the mitochondrial *COI* gene. Standard curves were made between 10 ng and 0/064 picogram. Unknown samples of caviar cream DNA concentration ranges were 2 ng – 400 pg according to the standard curve and *ct* amplification curve, indicating these samples belong to sturgeon DNA. None of the irrelevant genomic DNA (human, yeast, and bacteria) was indistinguishable from the response of negative control when analyzed with the *COI* sturgeon's specific primer. Real-time PCR could detect picogram and nanogram levels of residual DNA in cosmetic products, and verification of caviar cream was approved for made by caviar extract.

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