DOI: 10.29326/2304-196X-2021-3-38-209-215

Comparative study of PCR test kits for ASFV DNA detection

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SUMMARY

The paper presents comparative test results of 12 domestically produced diagnostic kits/PCR test systems for DNA detection of the African swine fever virus with regard to the following parameters: completeness and correctness of instructions for use; labeling and package contents; convenience of using the kit; shelf life stability of reagents; stability of reagents after transportation and repeated freezing – thawing; batch-to-batch repeatability; sensitivity of various test materials and specificity of kits. The study of the instructions for use and kit contents revealed incompleteness of some instructions. It was noted that some manufacturers make serious errors in the instructions, which can significantly affect the interpretation of test results. It was also observed that there is insufficient control of the manufacturing process, which results in the production of faulty kits, as well as kits with poor-quality components and errors in the labeling. Thus, during the study, one kit showed its inactivity, demonstrating the absence of accumulation curves of the fluorescent signal during amplification of both positive controls and DNA of ASFV isolates. When the specificity was assessed, all the kits showed absence of non-specific reactions and acceptable sensitivity when testing various types of ASFV-containing material (blood, suspensions of pork spleen and pork casings used in sausage production). The stability test showed a sharp deterioration in the quality of operation of one kit within the shelf life period, and a significant decrease in the fluorescence signal was detected during repeated freeze – thaw cycles for another kit. Comparison of the repeatability results of different kit batches of the same manufacturer showed significant discrepancies for 41.5% of all kits. It was found that only 33% of the studied kits for ASFV DNA detection were compliant. The results of this study demonstrate the need for control of the manufactured diagnostic kits used in state programs for animal disease monitoring.

Keywords: PCR test system, African swine fever, sensitivity, specificity, stability

Acknowledgements: The study was carried out with the support of the Rosselkhoznadzor within the research work: "Study of quality parameters of domestic and foreign diagnostic PCR test kits used in laboratory veterinary practice in the Russian Federation in order to develop common requirements for quality parameters and methods of their control". The authors express their gratitude to the FGBI "ARRIAH" and the FSBSI FRCVM for providing the ASF virus samples.

For citation: Krasnikova M. S., Yatsentyuk S. P., Bryusova M. B., Kozlova A. D. Gergel M. A. Comparative study of PCR test kits for ASFV DNA detection. *Veterinary Science Today*. 2021; 10 (3): 209–215. DOI: 10.29326/2304-196X-2021-3-38-209-215.

Conflict of interest: The authors declare no conflict of interest.

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УДК 619:578.842.1:616-076

Сравнительное исследование ПЦР-наборов для выявления ДНК вируса АЧС

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РЕЗЮМЕ

В работе представлены результаты сравнения 12 отечественных диагностических наборов/ПЦР-тест-систем для выявления ДНК вируса африканской чумы свиней по таким показателям, как полнота и грамотность инструкций по применению; маркировка и комплектация; удобство использования наборов; стабильность работы реагентов в течение срока хранения; стабильность реагентов после транспортировки и многократного замораживания – оттаивания; межсерийная сходимость; чувствительность при тестировании различного материала и специфичность наборов. Изучение инструкций по применению и комплектации наборов выявило неполноту некоторых инструкций. Отмечено, что отдельные производители допускают в инструкциях серьезные ошибки, которые могут существенно повлиять на интерпретацию результатов исследования. Также отмечена недостаточность контроля производственного процесса, результатом которой является выпуск неработоспособных наборов, а также наборов с низким качеством компонентов и ошибками в их маркировке. Так, при проведении исследования один набор показал свою неработоспособность, демонстрируя отсутствие кривых накопления флуоресцентного сигнала как при амплификации положительных контролей, так и ДНК изолятов вируса АЧС. При оценке специфичности все наборы показали отсутствие неспецифических реакций и приемлемую чувствительность при тестировании различных типов материала (крови, суспензий свиной селезенки и черевы свиной, используемой при производстве колбасных изделий), содержащих вирус АЧС. Проверка стабильности показала резкое ухудшение качества работы одного набора в пределах срока годности, для другого набора выявлено существенное снижение уровня флуоресцентного сигнала при многократном замораживании – оттаивании. Сравнение сходимости результатов работы разных серий наборов одного производителя показало существенные расхождения для 41,5% наборов. Установлено, что лишь у 33% рассмотренных наборов для выявления ДНК вируса АЧС отсутствуют какие-либо недостатки. Результаты проведенной работы демонстрируют необходимость контроля выпускаемых диагностических наборов, используемых в государственных программах мониторинга заболеваний животных.

Ключевые слова: ПЦР-тест-система, африканская чума свиней, чувствительность, специфичность, стабильность

Благодарность: Работа выполнена при поддержке Россельхознадзора в рамках научно-исследовательской работы по теме: «Изучение параметров качества отечественных и зарубежных диагностических ПЦР-наборов, применяемых в лабораторной ветеринарной практике на территории РФ, с целью разработки единых требований к параметрам качества и методам их контроля». Авторы выражают благодарность ФГБУ «ВНИИЗЖ» и ФГБНУ ФИЦВиМ за предоставленные образцы вируса АЧС.

Для цитирования: Красникова М. С., Яцентюк С. П., Брюсова М. Б., Козлова А. Д., Гергель М. А. Сравнительное исследование ПЦР-наборов для выявления ДНК вируса АЧС. *Ветеринария сегодня*. 2021; 10 (3): 209–215. DOI: 10.29326/2304-196X-2021-3-38-209-215.

Конфликт интересов: Авторы заявляют об отсутствии конфликта интересов.

Для корреспонденции: Яцентюк Светлана Петровна, кандидат биологических наук, заведующий отделом генодиагностики инфекционных болезней животных отделения биотехнологии ФГБУ «ВГНКИ», 123022, Россия, г. Москва, Звенигородское шоссе, 5, *e-mail: pcr-lab@vgnki.ru.*

INTRODUCTION

African swine fever (ASF) is a highly contagious porcine disease caused by the virus of *Asfarviridae* family that affects both domestic and wild pigs of all ages. ASF causes serious economic and production losses and is listed in the Terrestrial Animal Health Code of the World Organization for Animal Health (OIE) as a notifiable disease.

Given that no effective treatment and vaccine currently exist for the disease, ASF prevention largely depends on timely disease outbreak containment and eradication. In the Russian Federation, both domestic pigs and wild boars are annually subjected to diagnostic testing. According to the data of the reports (Form 1-vet A) summarized by the FGBI "Veterinary Centre" more than 670 thousand tests were carried out in 2019 and 473 thousand tests were carried for 9 months of 2020. In 2020, according to the Information Analysis Centre of the Rosselkhoznadzor Department for Veterinary Surveillance, 161 ASF outbreaks in domestic pigs and 110 ASF outbreaks in wild boars in the Russian Federation were reported and notified to the OIE [1].

Currently, polymerase chain reaction (PCR) is widely used for ASF diagnosis. Veterinary laboratories commonly use domestically produced reagent kit (test-kits) for PCR tests. The laboratories carrying out diagnostic tests in the framework of the official programmes shall be accredited by the Federal Service for Accreditation (RusAccreditation). Documents laying down test procedures and test methods shall be indicated in the scope of accreditation of the testing laboratory. Often such documents present themselves instructions to the test-kits or reagent kits and the laboratories shall strictly follow such instructions. The problem is that current diagnostic test systems do not undergo mandatory official registration and certification, there is no list of requirements for them, the instruction text is not approved and the kits are not subjected to independent checks for their quality. This could result in release of substandard kits that in case of their use in tests could be responsible for ineffective diagnosis.

There are several types of PCR test systems/kits for ASF diagnosis on the Russian PCR diagnostica market that contain various components including those with electrophoretic detection and real time hybridization-fluorescent detection of amplification products; full-optional kits containing both PCR reagent kit and DNA extraction kit and, in case of electrophoretic detection, electrophoresis

kit, as well as kits designed for PCR only. For the latter kits, the manufacturer could provide recommendations in the instructions on the nucleic acid extraction kit to be used together with its PCR kit or extraction method to be used or indicate that any kit for nucleic acid extraction can be used. PCR control samples are essential kit components. However, there is also no uniformity here: some kits include an endogenous and/or exogenous internal control sample (ICS) allowing control of sample collection procedure and extraction quality, other kits do not contain ICS that reduces the reliability of diagnostic testing. No comparative assessment of the diagnostic kits for ASFV DNA detection available on the Russian diagnostica market has been carried out yet.

The study was aimed at comparative assessment of domestically produced diagnostic PCR kits for ASFV DNA detection for the following parameters: completeness and correctness of instructions for use; labeling and kit contents; convenience of the kit usage; stability of the reagents during the shelf life declared by the manufacturer; stability of reagents after transportation and repeated freezing – thawing; batch-to-batch repeatability when different batches of the kits from the same manufacturer were used; detection limit (sensitivity) when the kit was used for testing various materials and specificity of kits.

MATERIALS AND METHODS

The PCR kits for ASFV DNA detection produced by the following Russian manufacturers were assessed: the Federal Budget Institution of Science "Central Research Institute of Epidemiology" of the Federal Service for Customers' Rights Protection and Human Wellbeing (Rospotrebnadzor), "Lytech" Co. Ltd., Syntol JSC, "FBio" Co. Ltd., Innovative Diagnostic Systems Ltd., "VetFactor" Ltd., "VMT" Ltd., "Vetbiochem" Ltd., "Technology Centre" Ltd., "Organic-Test" Ltd. The assessed kits and their manufacturers were coded when the study results were discussed.

VetMAX ASFV Detection Kit (Thermo Fisher Scientific Inc., USA) validated and certified by the OIE (approval number: 20200114) was used as a reference diagnostic test-system.

A panel of the following 38 different samples, including bacterium and virus strains, was used for testing of the kits for their specificity: 'Skif' strain of Aujeszky's disease virus, 'Ilyinogorsky' strain of porcine transmissible gastroenteritis virus, 'IS' strain of porcine epidemic diarrhea virus, 'KS' and 'LK-VNIIVViM' strains of classical swine fever virus, 'VL90-94' strain of porcine parvovirus, 'G10 P11' strain of rotavirus, 94881 strain of porcine reproductive and respiratory syndrome virus, 1010 strain of porcine circovirus, *Bordetella bronchiseptica* ATCC 4617, *Brachyspira pilosicoli* ATCC 51139, *Brucella abortus* 82 ser. 022, *Brucella suis* 1330 strain, *Campylobacter jejuni* '70.2T strain', *Chlamydia psittaci* 'LS-87 strain', *Clostridium perfringens* 'Amo' strain, *Escherichia coli* 0157:H7, *Erysipelothrix rhusiopathiae* ATCC 8139, *Haemophilus parasuis* 'Uralsky' strain, *Histophilus somni* ATCC 700025, *Klebsiella pneumoniae* 'K2 5055' strain, *Lawsonia intracellularis* 'MS B3903' strain, *Leptospira interrogans* Pomona 'VGNKI-6 strain', *Listeria monocytogenes* 'USKHI-6' strain, *Mycobacterium avium* 'D4' strain, *Mycobacterium bovis* 1414 strain, *Mycobacterium paratuberculosis* 19698 strain, *Mycoplasma hyopneumoniae* 'J' strain, *Mycoplasma hyorhinis* field isolate, *Pasteurella multocida* ATCC 43137, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* 'Dublin 6' strain, *Shigella sonnei* ATCC 25931, *Staphylococcus aureus* 'VKPMV 6646' strain, *Streptococcus pyogenes* ATCC 19615, *Yersinia enterocolitica* 'My O3 BNIPCHI Microbe' strain, *Yersinia pseudotuberculosis* 192 strain, as well as porcine genome DNA and a panel of 6 suspension samples containing ASF virus obtained from the FGBI "ARRIAH" collection (Kaliningrad 10/17, Oryol 07/18, Arm07, Krasnodar 07/17, Leningrad 02/19 isolates) and the Federal Research Centre for Virology and Microbiology ('Stavropol 01/08' strain).

The sensitivity (limit of detection) of the full-optional kits comprising both PCR reagent kit and DNA extraction kit in different matrices – biological materials (blood, spleen) and porcine small casing used for sausage production – was assessed for the full-optional kit as a whole. 'A DNA/RNA-S-Factor' DNA extraction kit ("VetFactor" Ltd., Russia) was used for the assessment of the test-kits intended for PCR assay only without any recommendations for DNA extraction in the instructions for their use. Ten-fold serial dilutions of ASFV Leningrad 02/19 isolate (initial titre 6.2 lg HAU_{50}/cm^3) were prepared in 10% porcine spleen and casing suspensions and in blood. DNA extraction and subsequent PCR were carried out in triplicate for each dilution of the materials of each type.

Serial dilutions of ASFV Kaliningrad 10/17 isolate (initial titre 5.8 lg HAU_{50}/cm^3) prepared with saline solution were used for comparative assessment of the amplification kits (without DNA extraction stage) for their sensitivity. 'Ribo-prep' kit (Federal Budget Institution of Science "Central Research Institute of Epidemiology" of the Rospotrebnadzor) was used for DNA extraction. Extracted DNA was used for PCR amplification using kits of different manufactures. Each virus dilution was tested with PCR in triplicate.

Nucleic acid was extracted from ten-fold dilutions of ASFV Leningrad 02/19 isolate prepared with saline solution for comparative assessment of the effectiveness of DNA extraction using extraction kits from different manufacturers. PCR was carried out with 'VetMAX ASFV Detection Kit' (Thermo Fisher Scientific Inc., USA), reference reagent kit.

DNA amplification was carried out with 'CFX96 C1000 Touch' (Bio-Rad Laboratories Inc., USA), 'Rotor-Gene Q' (QIAGEN GmbH, Germany) and 'Tertsik' (DNA-Technology LLC, Russia) depending on the PCR product detection method indicated by the kit manufacturer.

For testing reagent kits for their stability, results of positive and negative control amplification were assessed every three months within the kit shelf life period. To test reagents for their resistance to transportation temperature conditions recommended by their manufacturers as well as to repeated freezing – thawing every reagent was divided into three equal parts. The first part was kept at temperature recommended by the manufacture for the reagent storage period. The second part was subjected to multiple freeze-thaw cycles (up to 15 cycles). The third part was kept in a thermo insulating plastic foam box placed in ice for maximum transportation period indicated by the manufacturer. Comparative assessment of positive and negative control amplification results for each diagnostic kit was carried out in several repeats upon the testing completion.

Identical reagent kits of different batches were compared for their performance to assess batch-to-batch

reproducibility taking into account data of comparative assessment of positive controls and dilutions thereof as well as ASFV DNA-containing samples for their amplification.

Repeatability- and reproducibility-related precision was determined as closeness of the measurements obtained with multiple analyses of the same sample [2, 3]. The arithmetic mean of the threshold cycle Ct, the standard deviation and the coefficient of variation were calculated. The set of the obtained data was considered homogeneous when the coefficient of variation was less than or equal to 10%; sufficiently homogeneous – when the coefficient of variation was within 10–20%; sufficiently heterogeneous – when the coefficient of variation was within 20–33%; heterogeneous – when the coefficient of variation was higher than 33% [4].

RESULTS AND DISCUSSION

Twelve test-kits from 10 manufacturers were included in the study: 10 PCR test kits with real time hybridization-fluorescent detection and 2 PCR test kits with electrophoretic detection.

The study of the instructions for use to the reagent kits (test-systems) has revealed that some manufactures pay insufficient attention to their preparation as well as to their component labeling. There are serious errors in the instructions for use including those that contradict the regulations for the laboratories that use nucleic acid amplification methods [5]. Such errors could seriously affect the test results interpretation and lead to false positive or false negative results. Some instructions lack data on the sample preparation for DNA extraction. For test-kit No. 12, the manufacturer replaced the instruction for use by the leaflet that contained information on DNA amplification procedure and on interpretation of the results but lacked the description of the kit components as well as the reagent storage and transportation conditions.

Analysis of the kits' contents and ergonomics has showed that some manufacturers incorrectly estimate the control sample amounts without regard for the probable tests of single biological material samples in the laboratory.

Tests of all test-kits for their specificity showed that they, except for test-kit No. 12, correctly detected ASFV DNA in all tested samples containing ASFV isolates recovered on the territory of Russia at different times.

Amplification kit No. 12 demonstrated its malperformance when nine reagent kits of the said manufacturer were tested by different operators using different machines at different times. No fluorescent signal against ASFV was detected during the amplification of DNA extracted from the virus isolates as well as positive controls included in the test-kits. Therefore, PCR test-kits from the said manufacturer were excluded from the other tests.

Results of assessment of the amplification kits for their sensitivity without taking into account DNA extraction stage as well as assessment of the sets of primers for their sensitivity during testing of the DNA extracted from the materials of different types (blood, porcine spleen and casing suspensions) are summarized in Table 1.

Decrease in the virus detection limit was found for some kits when they were used for tests of the porcine

Table 1
Comparative results of sensitivity assessment of amplification kits when testing various ASFV-contaminated materials

Designation of coded test-system/kit	Sensitivity of ASFV DNA amplification kits			
	without DNA extraction stage	when DNA is extracted from the materials of different types		
	detected titre of ASFV Kaliningrad 10/17 isolate in saline solution (initial titre 5.8 lg HAU_{50}/cm^3)	detected titre of ASFV Leningrad 02/19 isolate (initial titre 6.2 lg HAU_{50}/cm^3)		
		in porcine blood	in porcine spleen suspension	in porcine casing suspension
No. 1	0.8	3.2	4.2	3.2
No. 2	0.8	3.2	3.2	3.2
No. 3	0.8	2.2	2.2	2.2
No. 4	0.8	2.2	2.2	1.2
No. 5	1.8	4.2	3.2	2.2
No. 6	1.8	3.2	4.2	3.2
No. 7	1.8	3.2	3.2	2.2
No. 8	1.8	3.2	3.2	4.2
No. 9	1.8	4.2	4.2	4.2
No. 10	1.8	3.2	3.2	2.2
No. 11	2.8	3.2	3.2	4.2

Table 2
Comparative test results of different batches of kits with real time hybridization-fluorescence detection

Designation of coded test-system/kit	Amplification of positive control sample detected as	Batch 1, Ct	Batch 2, Ct	Standard deviation	Coefficient of variation, %
No.1	ASF	17.36	18.03	0.34	1.89
	ICS	19.28	19.50	0.11	0.57
No. 2	ASF	26.89	28.87	0.99	3.55
	ICS	29.35	22.55	3.40	13.40
No. 3	ASF	19.22	17.35	0.93	5.11
	ICS	20.23	19.51	0.36	1.81
No. 4	ASF	32.68	34.14	0.73	2.18
	ICS	31.35	32.14	0.40	1.24
No. 5	ASF	23.15	23.24	0.04	0.19
	ICS	20.44	20.37	0.04	0.17
	exogenous ICS	21.76	21.83	0.03	0.16
No. 7	ASF	23.68	16.61	3.54	17.55
No. 9	ASF	16.87	20.32	1.73	9.28
No. 10	ASF	19.69	19.87	0.09	0.46
	ICS	24.17	26.34	1.09	4.30
No. 11	ASF	absence of detection	10.15	–	–

materials of different types. This fact becomes important in case of testing of porcine food products (sausages, minced meat) for African swine fever in which the virus concentration could be small. Therewith, some of amplification kits (No. 2, 3, 9) showed similar sensitivity regardless of the type of tested material. In general, it should be noted that all amplification kits demonstrated suitable sensitivity. Therefore, number and type of tested samples as well as contamination risk should be considered when choosing among them.

Since the stage of nucleic acid extraction plays an important role in the PCR testing, effectiveness of the DNA extraction with the kits of different manufacturers was assessed separately. Extraction effectiveness was assessed by comparing PCR results obtained using the reference kit, 'VetMAX ASFV Detection Kit'.

The effectiveness of DNA extraction with kits No. 7–9 was found to be lower than that one of the other tested kits. ASF virus extracted with the said kits was detected with 'VetMAX ASFV Detection Kit' at a titre of 4.2 lg HAU_{50}/cm^3. Whereas, ASF virus extracted with kits No. 1–6 and 10 was detected with 'VetMAX ASFV Detection Kit' at a titre of 2.2 lg HAU_{50}/cm^3. Despite of malperformance of PCR kit No. 12 the assessment showed its sufficiently high DNA extraction effectiveness when different extraction kits from this manufacturer were used: all reagent kits based on different DNA extraction methods demonstrated similar effectiveness (detected virus titre 3.2 lg HAU_{50}/cm^3).

It should be noted that all assessed DNA extraction kits complied with their intended use and allowed effective DNA extraction when they were used for tests of the materials of animal origin.

Tests of the kits for their stability during their shelf life showed drastic decrease in kit No. 8 performance at the last time point of storage (the 12th month of storage). Other kits demonstrated high and sufficient consistency of PCR results during the whole storage period with variation coefficient of 10% or within 10–20%.

Assessment of the kit components for their resistance to multiple freezing – thawing and storage under transportation conditions showed high stability of ten out of eleven kits. For kit No. 7, two-fold fluorescent signal decrease during amplification product detection was found after the kit reagents were subjected to multiple freezing – thawing as compared with the fluorescent signal obtained when aliquots of the original reagents were used.

Comparative testing of different batches of the kit from same manufacturer revealed batch-to-batch discrepancies in the kit performance for five out of eleven tested kits. Kit No. 6 with electrophoretic detection of amplification products showed high consistency of the results for two different batches, whereas kit No. 8 with analogous

detection method demonstrated discrepancies for positive extraction control included in the kit as a component: for one kit batch amplification product was detected when ten-fold dilution of this component was used and for other kit batch – amplification product was detected when the non-diluted component was used. This could be indicative of shortcomings during the control sample production and presence of large number of PCR inhibitors in the reagent of the first batch of the kit.

Table 2 shows results of the comparative testing of positive controls included in the real time hybridization-fluorescent detection PCR reagent kits for ASFV DNA detection.

For test-kit No. 7, the difference in Ct values when positive control was amplified with different batches of the kit was 7 (coefficient of variance higher than 10%); whereas, ASFV DNA amplification results were almost identical. Also, malperformance of internal control amplification system was revealed for one batch of the kit. Generally, this is indicative of unstable quality of control samples, set of primers and probes of the said manufacturer.

Comparison of different batches of kit No. 2 revealed the 4-fold difference in fluorescent signal during detection of positive sample amplification products (coefficient of variance higher than 10%).

Differences in PCR internal control amplification were revealed for different batches of kit No. 10: difference in Ct values when PCR internal control was detected was more than 14 (coefficient of variance was higher than 25%) that was indicative of unstable production quality of that component of the kit.

Low repeatability of the results was demonstrated for different batches of kit No. 11: the kits of one batch showed good performance whereas kits of other batch (three kits of the batch were tested by different operators) did not demonstrate exponential increase in fluorescent signal intensity curves both for positive controls included in the test-kits and for control samples containing ASF virus.

CONCLUSION

The study shows that only four out of twelve assessed reagent kits for ASFV DNA detection from three different manufactures of PCR test systems for veterinary use have no disadvantages impeding their maximum effective use.

It is noted that some PCR test-kit manufacturers pay insufficient attention to the drawing-up of the instruction for their kit use and to the kit contents. Insufficient control of the kit production process results in labelling incompliance, poor quality of the components and, finally, malperformance of control samples, reagents and the whole test-kit. It was shown that sensitivity of the same kits used for tests of different materials indicated in their instructions for use could differ by two orders of magnitude. Kits of all manufacturers demonstrated absence of non-specific reactions; tests of the kits for their stability and batch-to-batch repeatability of the results showed that only five out of twelve tested diagnostic kits complied with the said parameters.

Currently, comparative assessment of diagnostic test systems is carried out only in the laboratory or by the provider during analysis of proficiency testing runs. The study results demonstrate the importance of official registration and regular control of the manufactured diagnostic kits used for official programmes on animal disease monitoring. Similar regulatory procedures for diagnostic kit market exist in the EU, USA and Canada. Analysis of these procedures has showed that, first, the following is taking into account during veterinary diagnosticum assessment: its compliance with the intended use, specificity, sensitivity, reproducibility of the results obtained with the reagent kit. There is a comparable procedure in the Russian Federation: procedure for medical device registration aimed at marketing of quality and safe products in Russia. Development of similar procedure for official control of veterinary diagnostic kits is a currently important task.

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Received on 09.03.2021
Approved for publication on 26.05.2021

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