EXPERIMENTAL / LABORATORY STUDIES

Analysis of Cell-Free Fetal DNA from Maternal Plasma and Serum Using a Conventional Multiplex PCR: Factors Influencing Success

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Abstract: Recent technology enables the use of cell-free fetal DNA in maternal plasma and serum for noninvasive prenatal genetic diagnosis. This study was designed to evaluate factors most likely to influence the success of a simple, cost efficient, reliable and replicable conventional PCR technique in the clinical routine of prenatal genetic diagnosis of selected cases. The results strongly suggest that DNA extraction and PCR cycle optimization are 2 major success-limiting steps and the maternal plasma is a better choice over serum for DNA extraction for such prenatal genetic diagnosis. In addition, the use of a ready-to-use PCR mixture containing heat-activated Taq polymerase significantly reduced the risk of nonspecific amplification and of primer dimerization formed at low temperatures during PCR setup and the initial PCR cycle eliminating false positive results and insufficient PCR amplification, respectively. Thus the ease, rapidity and effectiveness shown by the presented system requiring only optimization of routine PCR procedure and no additional sophisticated equipment could theoretically reduce the cost and number of invasive procedures required for prenatal diagnosis of X-linked recessive genetic disorders and of fetal RhD status.

Key Words: Noninvasive prenatal diagnosis, maternal plasma, maternal serum, fetal gender determination, conventional multiplex PCR

Introduction

Recent technology enables the use of both intact fetal cells (1-3) and cell-free fetal DNA (4-13) in maternal plasma and/or serum for noninvasive prenatal genetic diagnosis. However, circulating fetal cells in maternal blood are extremely rare and the techniques used for the isolation, enrichment and processing of these cells are very time consuming and intensive. Thus, the use of this approach has been limited in clinical routine. On the other hand, cell-free fetal DNA in the maternal plasma and/or serum offers a promising noninvasive alternative for fetal gender, which can be used for clinical pre-testing for X-linked genetic diseases, and RhD status determination (6,14-16).

The concentration of fetal DNA in maternal plasma was found to be much higher than that present in the cellular fraction (4). Lo et al. (5) showed that during early pregnancy fetal DNA concentrations were, on average, 3.4% of the total DNA in the maternal plasma and 0.13% in serum. Even though the highest concentration of fetal DNA seems to be present in maternal plasma, some researchers have had better specificity and sensitivity results with maternal serum samples (10,17,18), whereas others suggested using the maternal plasma (4,8,13). Comparable results with both maternal plasma and serum have been reported when highly sensitive techniques, such as real-time quantitative polymerase chain reaction (PCR), are used for the determination of

fetal gender or RhD status (19-21). However, expensive equipment renders their application difficult in a routine setting. Thus, especially for diagnostic facilities with limited financial resources, a simple, cost efficient, reliable and replicable conventional PCR technique may be an alternative solution to utilize this analysis in a routine clinical setting.

This study was designed to evaluate the factors most likely to influence the success of such a conventional PCR technique in the clinical routine of prenatal genetic diagnosis. The success rates of 2 different DNA extraction techniques, which were used in previous studies on both maternal plasma and serum samples in a conventional multiplex PCR setting, were compared. In addition, the importance of PCR cycle number optimization, use of a ready-to-use PCR mixture, and heat activated Taq polymerase for the accuracy of such prenatal genetic diagnosis has also been demonstrated in this study.

Materials and Methods

Patients and Sample Collection

Peripheral blood and amniotic fluid samples were obtained from 33 pregnant women undergoing amniocentesis at Pamukkale University Hospital, Department of Obstetrics and Gynecology. The study was approved by the Pamukkale University Medical Ethics Committee. All of the pregnant women participating in this study were selected at random and gave informed written consent before blood sampling. All underwent blood sampling before amniocentesis. At the time of blood collection, the gestational ages ranged from 16 weeks to 20 weeks (mean ± SEM, 17.67 ± 0.22).

Peripheral blood samples obtained from 5 healthy men were used to determine the PCR specificity and sensitivity, whereas peripheral blood samples obtained from 5 non-pregnant women served as negative controls. In each case, 2-3 ml of peripheral blood was collected into an EDTA-containing tube for plasma separation, and 7-8 ml of peripheral blood was collected into a plain tube containing no anticoagulant for serum separation. The blood samples were centrifuged at 3000g for 10 min, and the plasma and serum were carefully removed from their respective tubes and transferred into plain polypropylene tubes. The plasma and serum samples then underwent a second centrifugation at 3000g for 10 min, and these recentrifuged plasma and serum samples were

transferred into fresh polypropylene tubes. The samples were stored at -20 °C until further processing.

Amniotic fluid samples (1-2 ml per patient) collected from the same group of pregnant women undergoing amniocentesis were stored at +4 °C until further processing and were used for fetal sex determination. An amniotic fluid sample from each patient also underwent cytogenetic analysis for complete karyotyping.

DNA Extraction from Plasma and Serum Samples

DNA from plasma and serum samples was extracted using either the heat-based direct method (22) or the QIAamp DNA Blood Mini Kit method (Qiagen, Hilden, Germany) according to the "blood and body fluid protocol" (10,13), with minor modifications in each case.

Briefly, in the case of the heat-based direct extraction method 200 µl of plasma or serum in a 0.5 µl sterile Eppendorf tube was heated at 99 °C for 5 min. The heated sample was then centrifuged at maximum speed in a microcentrifuge, after which the clear supernatant was collected and 10 µl used for PCR. In the case of the QIAamp DNA Blood Mini Kit method, on the other hand, DNA was extracted from 400 µl of plasma or serum and was eluted into a final volume of 50 µl, and 5 µl was used as template for PCR analysis.

Each DNA solution extracted from the healthy men was serially diluted from 1:10 to 1:1000 to estimate the sensitivity of the PCR used. In addition, the 1:100 diluted samples from these control men were used for PCR cycle optimization study.

DNA Extraction from amniocytes

To extract DNA from amniocytes 500 µl of amniotic fluid was transferred to a 0.5 ml sterile Eppendorf tube. Cells were pelleted at 10,000x g for 2 min and the supernatant discarded. Pellets were resuspended in 30 µl of sterile water and heated at 99 °C for 5 min. Samples were cooled and centrifuged at maximum speed in a microcentrifuge, after which the clear supernatant was collected and 10 µl used for PCR.

PCR conditions

The Y-chromosome-specific sequence DYS14 (198 bp) and an autosomal locus, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (97 bp), were co-amplified in a multiplex PCR setting for the detection of fetal DNA in maternal plasma/serum and to assess the presence of DNA, respectively. We used the primers Y1.7 (5'-

CATCCAGAGCGTCCCTGGCTT-3') and Y1.8 (5'-CTTTCCACAGCCACATTTGTC-3') for DYS14 (23) and forward (5'-CCCCACACACATGCACTTACC-3') and reverse (5'-CCTAGTCCCAGGGCTTTGATT-3') for GAPDH (24). All PCR amplifications were performed in a total volume of 50 µl containing extracted DNA, 20 pmol of each primer (Y1.7, Y1.8, GAPDH forward, and GAPDH reverse), and 25 µl of HotStarTaq Master Mix (containing 2.5 units of HotStarTaq DNA polymerase, 1 x PCR buffer with 1.5 mM $MgCl_2$, and 200 µM of each dNTP (Qiagen, Hilden, Germany)). To test the importance of using a ready-to-use PCR mixture, plasma DNA samples from selected pregnant women were further PCR amplified with same components individually purchased (all from Qiagen, Hilden, Germany) and added to each reaction at the same concentrations as above and the results were compared. In addition, to test the importance of using a heat-activated Taq polymerase enzyme, plasma DNA samples from selected pregnant women were further PCR amplified with Taq PCR Master Mix (Qiagen, Hilden, Germany) containing the same components at the same concentrations as above, except for the HotStarTaq, which was replaced with a regular Taq polymerase.

The thermal cycling was as follows: initial activation of HotStarTaq DNA polymerase at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, with final extension at 72 °C for 10 min. The PCR cycle number was optimized using the same conditions with the modification of the cycle number, i.e. 30, 40, and 50 cycles. The PCR amplification products were separated by 2% agarose gel electrophoresis and visualized by exposure to ultraviolet light after ethidium bromide staining.

Samples were only tested once and the success rates of different DNA extraction techniques, i.e. the heat-based direct method or the QIAamp DNA Blood Mini Kit method, as well as the sources of the DNA extracted, i.e. plasma or serum, were compared to each other on the basis of the accuracy of single PCR results.

Anticontamination measures

Great care was taken to prevent PCR contamination (25). Aerosol-resistant pipette tips were used for all liquids. Separate areas were used for the extraction of DNA, the preparation of amplification reactions, the carrying out of amplification reactions, and the detection of the PCR products. A female author performed all DNA extraction and PCR amplification procedures.

Statistical analysis

Data on the success rates of different DNA extraction methods or different materials, from which DNA was extracted, were analyzed by testing for the difference between 2 population proportions.

Results

Using representative healthy control male and non-pregnant control female DNA samples it was demonstrated that in our multiplex PCR setup the DYS14 sequence represents the male gender, whereas the GAPDH sequence was used as an internal control for the presence of extracted genomic DNA (Figure 1A).

DNA samples from all 5 healthy males, either extracted via the heat-based direct method or the QIAamp DNA Blood Mini Kit method from either plasma or serum were PCR positive for both DYS14 and GAPDH sequences (data not shown). However, DNA samples from all 5 non-pregnant women for either extraction method from either material were PCR negative for the DYS14 sequence and PCR positive for the GAPDH sequence (data not shown). Thus, the gender specificity of either DNA extraction method from either material was 100%.

The sensitivities of these DNA extraction methods for the DYS14 sequence, on the other hand, did differ from each other for both materials (Figure 1B). The sensitivity (S) of even the best DNA sample extracted via the heat-based direct method from either material was within the dilution limits of 1:1000 < S < 1:100 (Figure 1B lower panel). However, that of an average DNA sample extracted via the QIAamp DNA Blood Mini Kit method for either material was always S < 1:1000 (Figure 1B upper panel).

To optimize the PCR cycle number used in this study a series of reactions with 1:100 dilutions of healthy control male DNA extracted from either material using either method was performed using different cycle numbers, i.e. 30, 40, and 50 cycles, whereas all other reaction conditions were kept constant. The results showed that 40 cycles gave the optimum results with 100% sensitivity and specificity (data not shown).
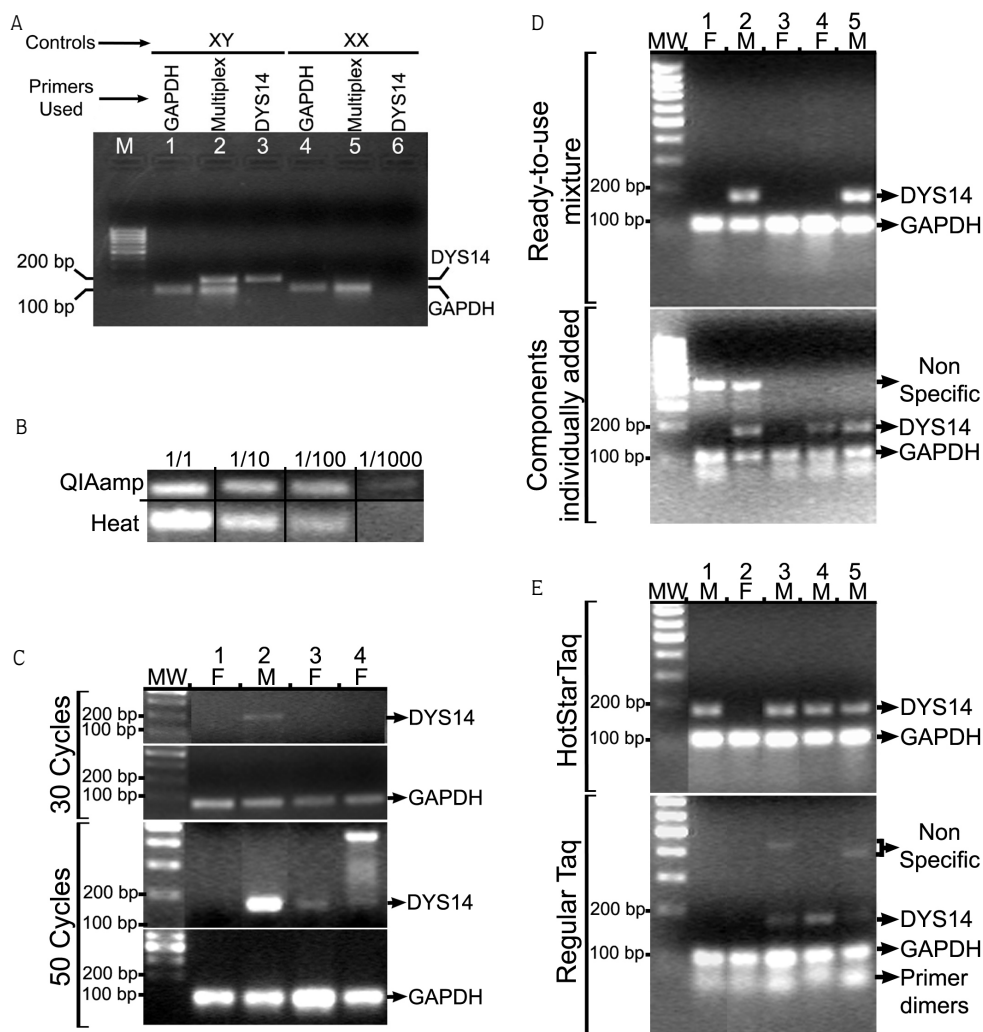
Figure. A) Multiplex PCR setup for DYS14 and GAPDH sequence. B) Analysis of the sensitivity of PCR used for gender determination. C) Analysis of the effect of PCR cycle number on the accuracy of prenatal genetic diagnosis results. D) Analysis of the effect of different PCR setup techniques on the accuracy of prenatal genetic diagnosis results. E) Analysis of the use of heat activated Taq polymerase versus regular Taq polymerase on the accuracy of prenatal genetic diagnosis results.

Cytogenetic analysis of cultured amniocytes revealed that 17 of the pregnant women were carrying a male fetus and the remaining 16 pregnant women were carrying a female fetus (Table). One of the female fetuses was cytogenetically detected with trisomy-13 (Table, patient 16). Optimized PCR results obtained with DNA extracted from amniocytes and from maternal plasma using the QIAamp DNA Blood Mini Kit method were in complete agreement with the cytogenetic analyses in terms of gender determination (Table).

All other optimized PCR attempts failed to obtain 100% accuracy for the diagnosis of fetal gender (Table). DNA extracted from maternal serum using the QIAamp DNA Blood Mini Kit method gave 31 accurate and 2 false negative (Table, patient 5 and 21) results. DNA extracted from maternal serum using the heat-based direct method gave 29 accurate results, 2 inconclusive results due to smear-like appearance (Table, patient 1 and 4), and 2 false negative (Table, patient 13 and 24) results. Finally, DNA extracted from maternal plasma using the heat-based direct method gave 29 accurate results, 1

Table. Fetal gender determination in pregnancies at risk of genetic disorders.

Patient No.	Week of gestation	Karyotype	DYS14 / GAPDH amplification					Risk
				Plasma		Serum		
			Amniocytes (A)	Kit (B)	Heat (C)	Kit (D)	Heat (E)	
1	20	46, XX	- / +	- / +	- / +	- / +	Smear	S
2	16	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	MA
3	18	46, XX	- / +	- / +	- / +	- / +	- / +	S+MA
4	16	46, XX	- / +	- / +	- / +	- / +	Smear	MA
5	19	46, XY	+ / +	+ / +	+ / +	- / +*	+ / +	MA
6	17	46, XY	+ / +	+ / +	- / +*	+ / +	+ / +	MA
7	18	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	S
8	16	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	MA
9	17	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	NT
10	16	46, XX	- / +	- / +	- / +	- / +	- / +	S
11	19	46, XX	- / +	- / +	- / +	- / +	- / +	S
12	18	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	S
13	20	46, XY	+ / +	+ / +	+ / +	+ / +	- / +*	DS
14	17	46, XX	- / +	- / +	- / +	- / +	- / +	MA
15	18	46, XX	- / +	- / +	- / -**	- / +	- / +	S
16	16	47, XX+13	- / +	- / +	- / +	- / +	- / +	S
17	18	46, XX	- / +	- / +	- / +	- / +	- / +	S
18	17	46, XX	- / +	- / +	- / +	- / +	- / +	S
19	17	46, XX	- / +	- / +	- / +	- / +	- / +	MA
20	19	46, XX	- / +	- / +	- / +	- / +	- / +	S
21	17	46, XY	+ / +	+ / +	+ / +	- / +*	+ / +	S
22	19	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	S
23	18	46, XX	- / +	- / +	Smear	- / +	- / +	MA
24	19	46, XY	+ / +	+ / +	+ / +	+ / +	- / +*	MA
25	17	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	S
26	18	46, XX	- / +	- / +	- / +	- / +	- / +	MA
27	16	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	S
28	19	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	S
29	17	46, XX	- / +	- / +	- / +	- / +	- / +	MA
30	20	46, XY	+ / +	+ / +	- / +*	+ / +	+ / +	S
31	16	46, XX	- / +	- / +	- / +	- / +	- / +	MA
32	17	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	S
33	18	46, XY	+ / +	+ / +	+ / +	+ / +	+ / +	S
Mean (±SEM): 17.68 (±0.25)		Accuracy:	100%	100%	88%	94%	88%	
Range:	16-20	Accuracy: Kit (B+D) = 97% versus Heat (C+E) = 88% [δ] (P < 0.03)						

S, Screening; MA, maternal age; NT, nuccal translucency; DS, history of child with Down Syndrome; *, false negative; **, no amplification; SEM, standard error of the mean.

[δ] Statistically different by the test for difference between 2 population proportions (P < 0.03).

inconclusive result due to smear-like appearance (Table, patient 23), 2 false negative (Table, patients 6 and 30) results, and 1 inconclusive result due to the absence of amplifiable genomic DNA (Table, patient 15).

To demonstrate the importance of PCR cycle number optimization for such prenatal genetic diagnosis studies, maternal plasma-DNA extracted via the QIAamp DNA Blood Mini Kit method from 4 male- and 4 female-fetus carrying pregnant women, whose PCR results with optimized 40 cycles were in 100% accordance with the results from cytogenetic testing, were further PCR amplified using either 30 cycles or 50 cycles. Results from 4 male-fetus carrying pregnant women with 30 cycles showed no false negative results. However, the DYS14 products were quite faint and difficult to detect (a representative sample is shown in Figure 1C, lane 2). On the other hand, results from 2 out of 4 female-fetus carrying pregnant women at 50 cycles showed false positive and/or non specific results (Figure 1C, lanes 3 and 4).

The use of a ready-to-use PCR mixture containing heat-activated Taq polymerase significantly reduced the risk of nonspecific and/or false positive amplifications and eliminated insufficient PCR amplification due to primer dimerization formed at low temperatures during PCR setup or the initial PCR cycle (Figure 1D and E, respectively). Again, maternal plasma DNA extracted via the QIAamp DNA Blood Mini Kit method from 4 male- and 4 female-fetus carrying pregnant women was tested. When PCR components were added individually to each reaction 2 out of 8 samples showed nonspecific products (Figure 1D, lanes 1 and 2) and 1 out of 4 female-fetus carrying pregnant women samples showed a false positive result for DYS14 product (Figure 1D, lane 4). On the other hand, when a regular Taq polymerase was used for PCR amplification all reactions showed significant primer dimerization (representative samples are shown in Figure 1e, lanes 1-5), 2 out of 8 samples showed nonspecific products (Figure 1E, lanes 3 and 5) and 1 out of 4 male-fetus carrying pregnant women samples showed a false negative result for DYS14 product (Figure 1E, lane 1). In addition, the remaining 3 accurate DYS14 products in the regular Taq group were quite faint and difficult to detect when compared to those in the HotStarTaq group (Figure 1E, lanes 3-5).

Discussion

This study was designed to compare the success rates of 2 different DNA extraction techniques, the heat-based direct method and the QIAamp DNA Blood Mini Kit method, used extensively in previous studies on both maternal plasma and serum samples in a conventional multiplex PCR setting to determine the best material and the protocol to be used for prenatal gender diagnosis. The crucial role of PCR optimization has also been demonstrated.

Our results indicated that the diagnostic accuracy of serum (94% with QIAamp DNA Blood Mini Kit extraction, and 88% with heat-based direct extraction) and plasma (100% with QIAamp DNA Blood Mini Kit extraction, and 88% with heat-based direct extraction) samples did not differ statistically from each other when compared within the same DNA extraction group ($P > 0.05$). On the other hand, the cumulative (plasma and serum samples together) accuracy of QIAamp DNA Blood Mini Kit extraction (97%) was significantly higher than that of heat-based direct extraction (88%; $P < 0.03$) suggesting that DNA extraction may indeed be one of the major success-limiting steps in such prenatal genetic diagnosis. The PCR results of serial dilutions of DNA extracted with either QIAamp DNA Blood Mini Kit or the heat-based direct method support this hypothesis. The sensitivity of even the best DNA sample extracted via the heat-based direct method from either plasma or serum was within the dilution limits of $1:1000 < S < 1:100$ (Figure 1B, lower panel). However, that of an average DNA sample extracted via the QIAamp DNA Blood Mini Kit method for either material was always $S < 1:1000$ (Figure 1B, upper panel). Thus, the QIAamp DNA Blood reagent set or equivalent is one of the best methods for extracting DNA from plasma or serum. Heat-based extraction, on the other hand, is cost efficient and very fast, although it seemed inadequate for some of the studied samples (Table).

In terms of the material utilized, this study indicated no significant difference between serum and plasma, although plasma samples scored the best, i.e. 100%, accuracy. The slightly better accuracy of plasma samples compared to that of serum samples may well be due to the concentration difference of cell-free fetal DNA contained in maternal plasma versus serum. Lo et al. (5) showed that during early pregnancy fetal DNA concentrations were, on average, 3.4% of the total DNA

in maternal plasma and 0.13% in serum. In this regard, our results are also in agreement with those of previous studies (4,8,13), which showed either no significant difference between plasma and serum sample accuracy (plasma being the more successful) (4), or plasma as a better choice for DNA extraction (8,13).

This study also highlights the significance of amplification cycle number optimization for such prenatal genetic diagnostic studies. As demonstrated here (Figure 1C), over-amplification of sample DNA increases the risk of false positive results due to nonspecific amplification. In addition, ready-to-use PCR mixtures containing all the necessary components except for the template DNA and primers significantly reduce the pipetting steps involved in the PCR setup, thus reducing the risk of sample contamination and nonspecific amplification (Figure 1D). Another significant contribution to the technique is the use of heat activated Taq polymerase, which is inactive at ambient temperatures preventing extension of nonspecifically annealed primers and primer-dimers formed at low temperatures during PCR setup and the initial PCR cycle as demonstrated in Figure 1E.

Based on the results of this study we concluded that the use of this optimized multiplex PCR approach would be practical in any clinical laboratory where a conventional PCR is available as an alternative to invasive prenatal diagnostic methods such as amniocentesis and chorionic villi sampling in cases of X-linked recessive genetic disorders. Thus, this could theoretically reduce the cost and number of invasive procedures required for prenatal diagnosis of X-linked recessive genetic disorders since they might be necessary for male fetuses only, significantly avoiding invasive procedures for female fetuses. This technique may also be used for the safe and rapid evaluation of fetal RhD status. It is also important to remember that 2 previous studies (10,13) have demonstrated the detection of cell-free fetal DNA from either maternal plasma (13) or serum (10) as early as 7th-10th gestational weeks, further strengthening the advantage of such prenatal diagnosis in terms of timing during pregnancy as an alternative to invasive techniques in selected cases.

Acknowledgment

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