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Development of a Peptide Nucleic Acid Array Platform for the Detection of Genetically Modified Organisms in Food

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Two previously developed platforms, a multiplex polymerase chain reaction (PCR) and a peptide nucleic acid (PNA) array, the former allowing for the simultaneous detection of five transgenes and two endogenous controls in food and feed matrices and the latter for the assessment of the identity of amplified PCR products, were combined in order to develop a PNA array device for the screening of genetically modified organisms (GMOs) in food. PNA probes were opportunely designed, synthesized, and deposited on commercial slides. The length of the probes as well as the distance of the probes from the surface were evaluated and found to be critical points. The most suitable probes were found to be 15-mer PNAs linked to the slide surface by means of two 2-(2-aminoethoxy)-ethoxyacetic acids as spacers. The device was tested on a model system constituted by flour samples containing a mixture of standards at known concentrations of transgenic material, in particular Roundup Ready soybean and Bt11, Bt176, Mon810, and GA21 maize: The DNA was amplified using the specific multiplex PCR method and tested on the PNA array. The method proposed was found to be able to correctly identify every GMO present in the tested samples.

KEYWORDS: PNA; array; multiplex PCR; GMO; soybean; maize

INTRODUCTION

The advancement of biotechnologies applied to the agrofood industry has brought about, during the past few years, an increasing number of genetically modified organisms to be introduced into the food chain at various levels. Although the regulatory approach to this matter is different depending on the different attitudes of the national governments, the European Union (EU) has established the rule of labeling foods containing genetically modified organisms (GMOs) above 0.9%. Moreover, according to the regulation 178/2002 recently introduced in the European market, traceability of foods and feeds all along the food chain must be guaranteed. To comply with the regulations and with the consumer requests, every step of the food production must now be certified, thus greatly increasing the number of analyses required by both the producers and the control laboratories.

Although several analytical methods have been proposed for the detection of GMOs in food (*1*), routine methods generally involve the amplification of specific DNA sequences by means of polymerase chain reaction (PCR) techniques (*2*), able to detect even small amounts of transgenes in raw materials and processed foods (*3*, *4*). To speed up these analyses, several multiplex PCR methods, which combining several primer pairs allows for the simultaneous detection of different target sequences, have recently been reported (*5*, *6*). In a previous work (*7*), we developed and validated a multiplex PCR method for the simultaneous amplification of Roundup Ready (RR) soybean and MON810, Bt 176, Bt11, and GA21 maize, able to reveal the presence of these transgenes in raw materials, feeds, and foodstuffs down to a content of 0.25% each.

Microarray-based hybridization analyses have emerged in recent years as powerful tools in biological and biomedical research allowing for the simultaneous identification of several nucleic acid sequences, thus offering the possibility of rapid and cost effective screening analyses. Oligonucleotide-based microarrays have also been developed to be used, often in combination with multiplex PCRs, for the specific detection of GMOs in different matrices: A cloth-based hybridization array system (*8*) was developed for the detection of transgenic corn and maize; a multiplex quantitative DNA array-based PCR (*9*) has been proposed for the quantification of transgenic maize in food and feed; and more recently, a ligation detection reaction universal array (*10*) has been developed for the parallel detection of five transgenic events.

In a previous work (*11*), we used peptide nucleic acids

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Table 1. Primer Composition of the Unbalanced Multiplex PCR

target	primer pair	concn (μM)
MON810	P-E35 for	0.05
	MON810 rev-Cy5	0.5
RR	P-E35s for	0.05
	RR rev-Cy5	0.5
Maize zein	MZ for-Cy5	0.5
	MZ rev	0.05
Soy lectin	SL for	0.05
	SL rev-Cy5	0.5
Bt 11	Bt 11 for-Cy5	0.5
	Bt 11 rev	0.05
Bt 176	Ev 176 for-Cy5	0.5
	Ev 176 rev	0.05
GA 21	GA21 for	0.05
	GA21 rev-Cy5	0.5

(PNAs), oligonucleotide analogues in which the sugar−phosphate backbone has been replaced by a pseudopeptide chain of *N*-aminoethylglycine monomers (*12*), to build up an array for the specific hybridization and detection of two DNA sequences belonging to the transgenic construct of RR soybean and its endogenous control, the lectin gene. On account of the high sequence specificity of PNA and affinity for the DNA (higher than DNA itself) (*13*, *14*), it was possible to detect the presence of a transgenic DNA sequence by using a 15-mer PNA probe opportunely designed and synthesized for the purpose. On account of their specific characteristics, PNAs have been proposed as valuable alternatives to conventional oligonucleotide probes for the development of more efficient and more stable microarrays (*15*). Several PNA-based biosensors have been developed so far (*16*), and in particular, array formats were used for the detection of DNA mutations in biomedical applications (*17*, *18*).

In this paper, we report the development of a PNA chip able to simultaneously identify, in a single and reliable experiment, four types of transgenic maize (MON810, Bt11, Bt 176, and GA21), one transgenic soybean (RR), and two endogenous controls (the zein gene for maize and the lectin gene for soybean) after a multiplex amplification. Each PNA probe was opportunely designed in order to specifically hybridize to a known DNA sequence present in each target sequence. The length of the probes and the distance from the slide surface were tested to establish the best conditions for hybridization. All of the probes synthesized were then spotted in an array format and tested in order to assess their specificity and selectivity in DNA recognition.

MATERIALS AND METHODS

Sample Preparation. DNA was extracted from commercially available standards purchased from Fluka (GM-free soybean flour, 83063; GM-free maize flour, 63195; 5% RR soybean flour, 44386; 5% Mon810 maize flour, 76182; 5% Bt11 maize flour, 65944; 5% Bt176 maize flour, 17111; and 5% GA21, not available as commercial standard, was prepared as laboratory reference material). The extraction was performed using the Wizard kit from Promega as described in the Swiss Food Manual (*19*). DNA samples for amplification were prepared by mixing a volume of extracted solution corresponding to 30 ng of DNA for each transgene. A double amplification was performed: The first amplification consisted of a multiplex PCR as described in our previous work (*7*); during the second amplification, 2.5 μL of amplified material was used as template for an unbalanced PCR in which the primer of each pair copying the target sequence was labeled with a Cy5 group; the concentrations of labeled and unlabeled primers were 0.5 and 0.05 μM respectively, as described in **Table 1**. Briefly, PCR conditions were as follows: final volume, 50 μL; reagent concentrations: template DNA, 200 ng, PCR buffer, 1X (Qiagen GmbH); $MgCl_2$, 3.5 mM; dNTPS, 0.4 mM each (Euroclone); primer mix, 1X; HotStartTaq DNA Polymerase, 0.15 U/μL (Qiagen GmbH); thermal cycler conditions: preincubation at 95 °C for 10 min; 40 cycles consisting of dsDNA denaturation at 95 °C for 50 s; primer annealing at 60 °C for 50 s; primer extension at 72 °C for 50 s; final elongation at 72 °C for 5 min.

Probe Design. The PNA sequence was first checked to minimize any secondary structure, which would result in a loss of hybridization efficiency, using the online available program Mfold (version 3.1) (*20*). The sequence specificity of the probes was first evaluated for each transgene using the BLAST homology search system from DDBJ (http://www.ddbj.nig.ac.jp/E-mail/homology.html), and then, every probe was checked in order to avoid its possible hybridization on any other nontarget region among those amplified by the multiplex PCR, by aligning its sequence to the sequences of all of the amplified products.

PNA Synthesis. The PNA oligomers were synthesized by solid phase synthesis on a 433A Peptide Synthesizer (Applied Biosystems) using the BOC strategy and HATU/DIEA coupling, as described in a previous work (*21*). Swelling, downloading, and cleavage of the PNAs from the resin were done manually.

For the study of the effect of spacers as linkers between the probe and the chip surface, the 2-(2-aminoethoxy)ethoxyacetic acid was used according to previous results obtained in a similar experiment (*22*). The crude products were purified by reversed phase high-performance liquid chromatography using a Phenomenex C18 peptide column (3 μm, 250 mm × 10 mm) with a binary gradient (flow rate, 4 mL/min); eluent A, water/TFA = 100:0.2; eluent B, water/acetonitrile/TFA = 60:40:0.2; and detector UV, 260 nm. The purified products were characterized by electrospray ionization mass spectrometry.

Array Preparation. "CodeLink Activated Slides" (Amersham Biosciences, 300011) were used as solid supports to which the amino-terminal group of the PNA probes was covalently linked. The deposition of the probes was carried out using a GMS 417 Arrayer (Genetic Microsystem) with a pin-and-ring deposition system. As far as the deposition protocol, the manufacturer's instructions were slightly changed in order to comply with the special requirement of the chemical structures of PNAs: In particular, a carbonate buffer (100 mM, pH 9.0) containing 10% acetonitrile and 0.001% sodium dodecyl sulfate (SDS) was used as the deposition buffer. Moreover, after every deposition, the pin-and-ring system was purged with water for 10 s and further washed with acetonitrile/water (1:1), to avoid dragging of the probes in subsequent depositions. The probes were coupled to the surface, and the remaining reactive sites were blocked by leaving the slides for 12 h in a humid chamber (relative humidity 75%) at room temperature, followed by a 30 min immersion in a glass rack containing a 50 mM solution of ethanolamine, 0.1 M TRIS, pH 9, prewarmed at 50 °C. The slides were washed twice with bidistilled water at room temperature and then slowly shaken for 30 min in plastic tubes containing a 4× saline−sodium citrate (SSC) and 0.1% SDS buffer prewarmed at 50 °C. Each slide was then washed with bidistilled water at room temperature and centrifuged in a plastic tube for 3 min at 800 rpm. Slides were then ready to undergo the hybridization protocol or could be stored in a desiccated chamber for future use. It has to be remarked that, since a fluorescent control probe was deposited to check the efficiency of the deposition step, all of the previously described operations were carried out away from direct light in order to prevent degradation of the Cy5 fluorophore.

Sample Hybridization. DNA samples to be tested were prepared by diluting 50 μL of the PCR product to a final volume of 65 μL and a final concentration of 4× SSC and 0.1% SDS buffer. Hybridization was performed by loading the samples to "in situ frame" chambers (Eppendorf, 0030 127.510) and leaving the slides under slow shaking for 2 h at 40 °C. After the hybridization step, all of the slides were treated individually to prevent cross-contamination. The slides were briefly rinsed with a 4× SSC solution prewarmed at 40 °C and then washed under slow shaking for 5 min with a 2× SSC, 0.1% SDS buffer prewarmed at 40 °C, followed by treatment for 1 min with 0.2× SSC and 1 min with 0.1× SSC at room temperature. The slides were then spin-dried for 5 min at 1000 rpm. It has to be noticed that all post-

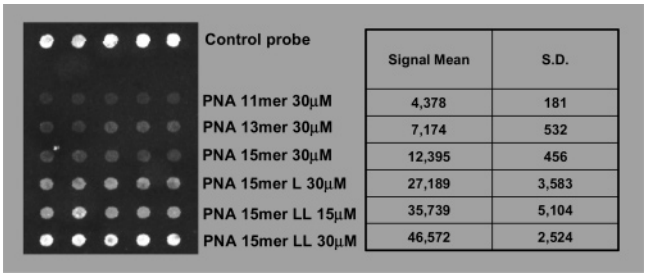

Figure 1. Evaluation of the effect of the probe length and the presence of a spacer on the hybridization PNA/DNA. All probes were hybridized with a 0.6 μM solution of a Cy5-labeled 79-mer synthetic DNA containing the target RR soybean sequence. Signal intensities reported refer to fluorescence arbitrary units. L: 2-(2-aminoethoxy)ethoxyacetic acid spacer.

hybridization steps were performed in a dark environment to prevent degradation of the Cy5 fluorophore used to label the target sequences.

Image Acquisition. The fluorescent signal deriving from the hybridization was acquired using a GMS 418 Array Scanner (Genetic Microsystem) at $\lambda_{ex} = 646$ nm and $\lambda_{em} = 664$ nm. To correctly compare the hybridization data, all of the images reported were acquired with laser power = 100 and photomultiplier gain = 40.

RESULTS AND DISCUSSION

The PNA probes were designed in order to target the seven DNA sequences amplified by the multiplex PCR (MON810 maize, 110 bp; RR soybean, 125 bp; maize zein, 139 bp; soybean lectin, 157 bp; Bt11 maize, 189 bp; Bt176 maize, 209 bp; and GA21 maize, 270 bp). The PNA sequences were chosen as to maximize their hybridization efficiency and to minimize any unspecific hybridization with their target strand and with any other nontarget sequence, following the parameters described in the Materials and Methods section.

In a previous work on the application of PNAs to the detection of transgenic DNA (*11*), it was found that the length of the PNA probe played an important role on the hybridization efficiency. Moreover, the best hybridization results were obtained when analyzing the target DNA single strand rather than the double strand PCR product.

To investigate the role of the length of the probe and the effect of the slide surface on its interaction with the target DNA, we performed further experiments with a set of probes newly synthesized, targeting a specific DNA sequence of RR soybean, of different lengths (11-, 13-, and 15-mer). One and two 2-(2-aminoethoxy)ethoxyacetic acid spacers were added (15-merL and 15-merLL, respectively) to the N-terminal group of the longest probe in order to evaluate the optimal distance from the slide surface. The PNA probes were spotted in a five replicate array format together with a Cy5-labeled DNA oligomer as a control for the deposition; the concentration of all probes was 30 μM apart from the 15-merLL PNA, which was also spotted at a lower concentration (15 μM). The array was hybridized with a 0.6 μM solution of a Cy5-labeled 79-mer synthetic DNA containing the RR soybean target sequence, to mimic the real sample conditions.

As it can be observed (**Figure 1**), all probes were able to correctly identify the target sequences although the hybridization response was greatly affected by the different parameters. In particular, as expected, by increasing the length of the probe from 11- to 15-mer, the hybridization yield was enhanced; however, the strongest effects were observed when the probes were elongated by one and two linkers: The hybridization yield strongly increased when adding one spacer group and further increased when two spacers were added. Particularly remarkable was the difference between one single spacer and two spacers: Indeed, the 15 μM 15-merLL PNA gave rise to a signal comparable to the 30 μM 15-merL PNA.

Table 2. PNA Probe Sequences and Target Regions[a]

probe	target	sequence (H–NH_2)
SL	soybean lectin	LL-GAT CAA GTC GTC GCT
MZ	maize zein	LL-TTT ATA GAT GTA TGC
MON810	MON 810 maize	LL-CGC TCA CTC CGC CCT
RR	RR soybean	LL-AAA CCC TTA ATC CCA
Bt11	Bt11 maize	LL-ATA TCT ACT GAC AAA
Bt176	Bt176 maize	LL-ACA CCT CGT TGC CGC
GA21	GA21 maize	LL-CGA ACT TCT TGT TGC

[a] L, 2-(2-aminoethoxyl)ethoxyacetic acid spacer group.

The sensitivity of a 15-merLL PNA in hybridizing its complementary DNA sequence was studied by spotting a set of probe concentrations (50, 40, 30, 20, and 10 μM) on 10 slides, which were then individually hybridized with concentrations of the target oligonucleotide corresponding to 0.8 μM, 0.4 μM, 0.2 μM, 0.1 μM, 50 nM, 25 nM, 12.5 nM, 6.75 nM, 1 nM, and 0.01 nM: The best hybridization signal (data not shown) was observed for the 30 μM PNA while no significant improvement was observed by further increasing the PNA concentration. The limit of detection, determined with oligonucleotide, was found to be 1 nM.

The design of all probes to be used to build up the PNA array took into account these preliminary results: All of the assays given ahead were performed with 15-mer PNA probes functionalized with two 2-(2-aminoethoxy)ethoxyacetic acid spacer groups at their N-terminal position and spotted at a concentration of 30 μM.

The probes chosen to build the final version of the array are shown in **Table 2**, together with their sequences and their targets. Seven replicates for each probe were spotted on the array; the deposition order was SL, MZ, MON810, RR, Bt11, Bt176, and GA21; a control probe was also spotted to check the deposition efficiency in order to better evaluate any eventual slide-to-slide variability.

DNA samples were prepared and amplified as described in the Materials and Methods section and individually tested on the slides. The compositions of the samples were as follows: sample 1, GMO free soybean; sample 2, GMO free maize; sample 3, 5% MON810 maize; sample 4, 5% RR soybean; sample 5, 5% Bt11 maize; sample 6, 5% Bt176 maize; and sample 7, 5% GA21 maize. As it can be observed (**Figure 2**), all of the samples were correctly identified by the complementary PNAs: in particular, while in sample 1 and 2 only the corresponding endogenous controls were identified as expected, for all of the other samples the correct couple of endogenous control and specific transgenic construct was identified for each transgene.

Once the specificity of the developed system was assessed, the selectivity was also tested in order to correctly characterize the composition of the amplified samples, using different combinations of GMOs. In particular, the results of the test for a sample made out of 5% RR soybean and 5% Bt176 maize are reported in **Figure 3**: As expected, the PNA array correctly recognized only the presence of soybean lectin and the RR construct for the transgenic soybean as well as the presence of maize zein and the Bt176 construct for the transgenic maize.

A further example is given in **Figure 4**, in which the flour sample contained four transgenes not including GA21 maize: Also in this case, the PNA array was able to selectively identify all of the transgenes present in the sample.

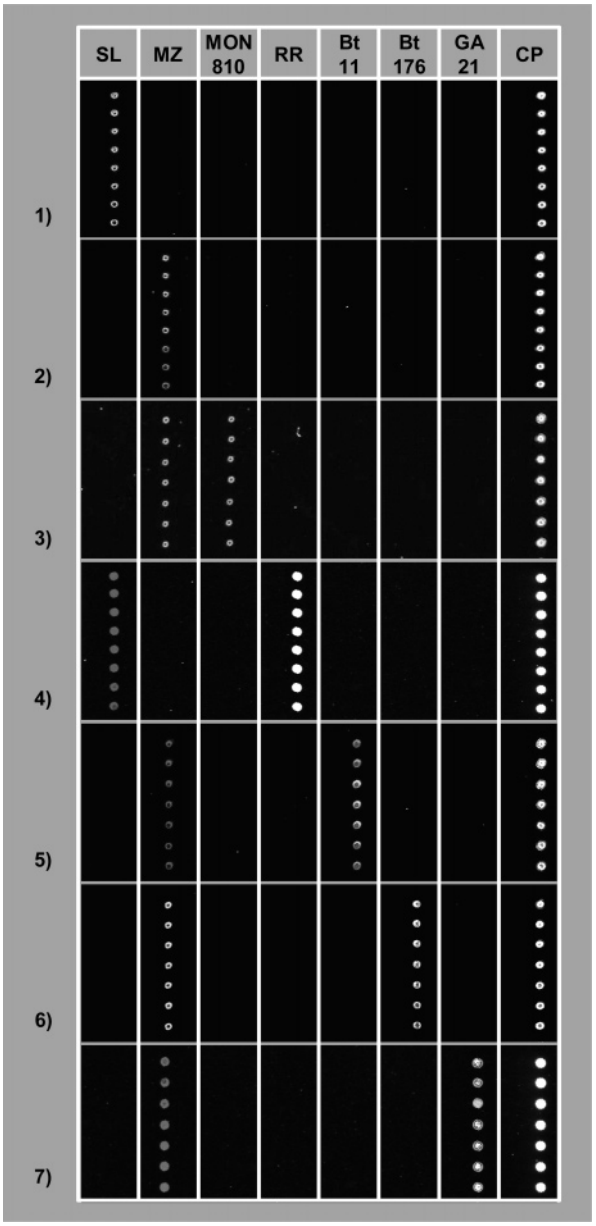

Figure 2. Specificity assessment of the PNA array. Each slide was hybridized with DNA and amplified twice by multiplex PCR, previously extracted from flour containing: 1, GMO free soybean; 2, GMO free maize; 3, 5% MON810 maize; 4, 5% RR soybean; 5, 5% Bt11 maize; 6, 5% Bt176 maize; and 7, 5% GA21 maize. The PNA probes were spotted, at concentration of 30 μM, as follows: SL (soybean lectin), MZ (maize zein), MON810 (MON810 maize), RR (RR soybean), Bt11 (Bt11 maize), Bt176 (Bt176 maize), and GA21 (GA21 maize).

However, it has to be noticed that the fluorescent signal after hybridization is not homogeneous for all of the probes utilized and does not reflect the relative quantities of the different target organisms. The variability was expected and can be essentially attributed to three different factors: (i) the different length of the target sequences (ranging from 110 to 270 bp), which can greatly affect the kinetics of hybridization; (ii) the different efficiency of amplification for the amplicons in the multiplex PCR system; and (iii) the different melting temperatures of the PNA/DNA duplexes. In particular, it has to be noticed that no information on the relative quantities of the single transgenes can be extrapolated from the array results since the amplified products are the result of a multiplex PCR, which was opportunely calibrated to harmonize the amplification of DNA

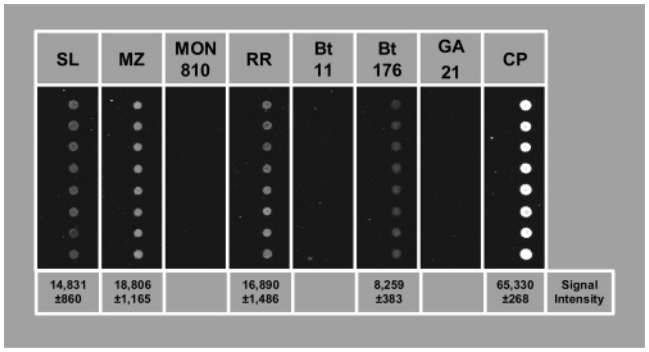

Figure 3. PNA array tested with a previously amplified flour sample containing 5% RR soybean and 5% Bt176 maize. Probes were deposited at a concentration of 30 μM. Signal intensities reported refer to fluorescence arbitrary units.

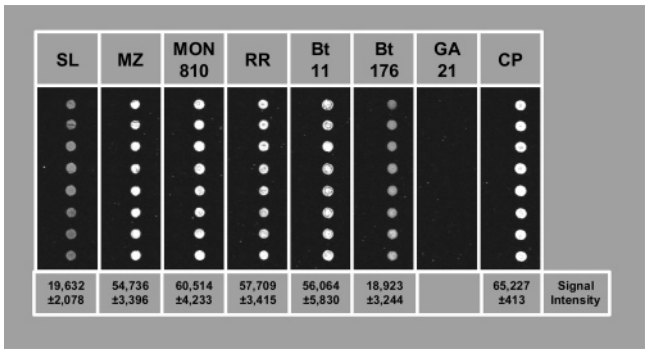

Figure 4. PNA array tested with a previously amplified flour sample containing 5% RR soybean, 5% MON 810 maize, 5% Bt11 maize, and 5% Bt176 maize. Probes were deposited at a concentration of 30 μM. Signal intensities reported refer to fluorescence arbitrary units.

material of seven different targets from a wide range of relative contents. Thus, the signal generated by hybridization of the reference gene and the specific transgenes, although rising from different amounts of template in the multiplex PCR, turns out to be of similar intensities on the microarray. However, because the method proposed here is purely qualitative, this should not be considered as a troublesome issue affecting the system.

Although the LOD for the PNA array tested with a complementary 15-mer DNA oligonucleotide was evaluated to be 1 nM (data not shown), the detection limits and applicability range of the whole system are strictly dependent upon the efficiency of the PCR amplification.

In this paper, PNAs are shown to be highly selective probes for the identification of single-stranded DNA in an unambiguous way. The protocol for linking the PNA probes to the commercially available slides has been modified from the original method devised for oligonucleotides, and spacers have been added to maintain the probes at a proper distance from the slide surface. The combination of this new array technology with the multiplex PCR method previously devised has allowed the construction of a PNA array for the simultaneous detection of soybean, maize, RR soybean, and MON810, Bt 176, Bt11, and GA21 maize. The method was found to be specific for identifying the presence of the target DNA sequences simultaneously present in complex model systems. At the moment, the method is purely qualitative; the detection is basically dependent on the previous amplification, so that the detection limits and applicability range are strictly dependent upon the efficiency of the multiplex PCR method employed. However, given the results previously published on the development of the multiplex PCR (*7*), we can conclude that the PNA array developed here

is a valid tool for screening the presence of GMOs in both raw materials and processed foods with a good specificity and sensitivity (limit of detection for the multiplex PCR = 0.25% for each GMO). In conclusion, the PNA array platform described here can be considered an interesting prototype for the development of robust methods for the simultaneous detection of several DNA targets along the food chain in a reliable way.

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