FOOD COMPOSITION AND ADDITIVES

Interlaboratory Transfer of a Real-Time Polymerase Chain Reaction Assay for Quantitative Detection of Genetically Modified Maize Event TC-1507

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A real-time polymerase chain reaction (QPCR) assay was developed for quantitative detection of a genetically modified (GM) maize event TC-1507 and modification to conventional PCR for qualitative purposes. Sequences 5'-flanking TC-1507 full-length insert were characterized and showed multiple rearrangements involving insert and maize chloroplast fragments. The event specificity of the TC-1507 assays was based on the detection of transgene and plant rearranged sequences found to 5' flank the insertion site.
They were fully specific and exhibited a limit of detection below 10 target copies, allowing consistent detection of 0.1% GM levels. The QPCR was highly linear and efficient and proved adequate for quantification of GM contents, aiming at the fulfillment of legal requirements established in the European Union (i.e., compulsory labeling of TC-1507 levels >0.9%). It satisfactorily determined TC-1507 contents on different matrixes and was successfully transferred to a different laboratory.

During the past decade, the development of biotechnology has revolutionized agriculture, e.g., by the introduction of genetically modified organisms (GMOs) with characteristics of interest. The GMOs have been extensively cultivated, reaching 81 million hectares in 2004; in the case of maize, GM varieties cover more than 23% of maize acreage (1), and their derived products have reached the global marketplace. As a consequence, some countries have established labeling and coexistence regulations (2–4), which require the development of adequate detection tools to be enforced.

Policies for labeling GMO foods differ among countries. As an example, in the European Union (EU) thresholds for unintended mixing of GMOs in non-GMs are 0.9% for EU-approved varieties and 0.5% for non-approved varieties with positive EU risk assessment (2). The most accepted GM detection methods rely on polymerase chain reaction (PCR) or real-time PCR (QPCR) techniques (5–7), and are based on the specific detection or quantification of transgenic and plant sequences. Screening methods pursue the amplification of sequences found in many different GMOs like the 35S promoter (8) and the nopaline synthase (NOS) terminator (9). Unequivocal detection of a GMO event can be achieved by amplifying edge fragments containing sequences of diverse origins that have been rearranged as a result of a particular transformation event, i.e., sequences from the transgenic construct and those at the insertion point at the genome of the host, such as MON810 maize (10, 11); BT176 and BT11 maize (12); Roundup Ready Soybean (13–15); StarLink maize (16); NK603 (17); and T25 (18) maize.

The transgenic maize line TC-1507 (Herculex I™ from Mycogen, Dow AgroSciences, Pioneer, Dupont) was genetically engineered by biolistic transformation and harbors 2 transgenes, *cry1Fa2* and *pat*. The delta endotoxin Cry1F confers maize resistance to European corn borer (*Ostrinia nubilalis*) and other lepidopteran pests, while phosphinothricin-*N*-acetyltransferase (PAT) makes TC-1507 tolerant to phosphinothricin herbicides such as Basta®, Rely®, Liberty®, and Finale®. The transformed *cry1Fa2* gene was from *Bacillus thuringiensis* with some modifications. Its transcription was directed by the promoter and a 5 untranslated region from the maize ubiquitin gene (*ubiZM1*), including the first exon and intron, and 3 termination/polyadenylation sequences derived from the *Agrobacterium tumefaciens* open reading frame 25 (ORF25). The *Streptomyces viridochromogenes pat* gene was

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transcriptionally regulated via promoter and terminator sequences derived from the 35S transcript of cauliflower mosaic virus (CaMV; www.agbios.com).

According to "Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed" (2), applicants for authorization of GMO events should propose appropriate methods for sampling, identification, and detection, which should be validated, where appropriate, by the Community Reference Laboratory (CRL). Within the frame of this regulation a TC-1507-specific QPCR assay was developed by Pioneer Hi-Bred, Dow Agrosciences, Mycogen Seeds and subsequently validated by the CRL Joint Research Centre (JRC, Ispra, Italy; http://gmo-crl.jrc.it). It targets the 3 recombination region of parts of the construct into the plant genome. An alternative assay targeting 5 flanking sequences would be highly advantageous for GMO control laboratories to grant adequate quantitative detection of TC-1507-containing samples, even in the case of laboratory cross-contaminations or GM varieties incorporating mutations that affect one QPCR target sequence. In addition, it is highly desirable to gain knowledge on the genomic regions surrounding the insertion site. Better information on the sequences that have been incorporated in commercialized transgenic lines and of the putative rearrangements that may have occurred at the pre-integration site (15) would presumably assist the evaluation of new GMOs and improve consumer confidence on new technologies.

Here we report the characterization of the TC-1507 5 flanking sequence and its use as target for a TaqMan®-based QPCR and conventional PCR assays. The methods prove to be fully selective and able to accurately quantify TC-1507 in a highly sensitive manner. In addition, we demonstrate transferability of the assays to one other laboratory.

EXPERIMENTAL

Plant Material

Powdered certified reference material (CRM) of maize 1507, Bt176, Bt11, MON810, GA21, NK603, and CBH-351 maize and Roundup Ready® (GTS40-3-2) soybean were obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and commercialized by Fluka (Buchs, Switzerland). Seed powder of maize Bt10 and its wild-type isogenic line were provided by the European Network of GMO Laboratories (ENGL). Leaves of *Sorghum bicolor*, *Oryza sativa*, *Hordeum vulgare*, *Brassica napus*, *Brasica oleracea*, *Solanum tuberosum*, *Lycopersicon esculentum*, *Helianthus anuus*, *Triticum aestivum*, and *Glycine max* were from plants cultivated at the IBMB-CSIC greenhouses.

Extraction of Genomic DNA

Genomic DNA was isolated from 0.1 g of each sample using the QIAGEN® DNeasy plant mini kit (QIAGEN GmbH, Hilden, Germany). DNA concentration was quantified by UV absorption at 260 nm using a NanoDrop ND1000 device (NanoDrop Technologies, Wilmington, DE). All samples showed a 260/280 nm ratio ranging from 1.7 to 1.9. Concentrations were further confirmed by agarose gel electrophoresis and ethidium bromide staining (19).

Characterization of TC-1507 5 Flanking Sequences

A TAIL-PCR approach (20) adapted to maize sequences (10) was used to amplify TC-1507 5 flanking sequences. Briefly, a first amplification was performed with 0.3 M primer Ubi279R (5 -AAGGAGAACACATGCACACT-3) and 5 M arbitrary primer 1 (AD2, 20) and followed a program alternating high and reduced stringency cycles. A 1 L amount of a 50-fold dilution of the primary PCR products was subjected to a second amplification with 0.3 M primer Ubi92R (5 -AACTGCACTTCAAACAAGTGTGA-3') and 5 M of the same arbitrary primer AD2, which also followed high and reduced stringency cycles. Secondary PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. Both genomic DNA extracted from 10% TC-1507 and 0% TC-1507 were simultaneously tested, and only PCR products appearing specifically in 10% TC-1507 samples were further analyzed. They were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ), cloned into the pCR®II-TOPO vector (Invitrogen Corporation, Carlsbad, CA), and sequenced with the secondary PCR specific primer Ubi92R (using the kit Big Dye® Terminator v.1.1; and the ABI 3100 genetic analyzer, Applied Biosystems, Foster City, CA). The obtained sequences were compared to publicly available sequences using the BLAST-N tool (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov).

TC-1507 Conventional PCR Reactions

PCR reactions were performed in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus Instruments, Emeryville, CA) and performed with the GeneAmp PCR core reagents (Applied Biosystems) in 20 L PCR mixture, including 1 buffer II, 1 mM MgCl2, 200 M dNTPs, 0.5 M TCF forward primer (5 GACGTCTCAATGTAATGGTTAACGA 3), 0.5 M TCR reverse primer (5 GGGTAACCGCTCTTCCAGTTGT 3), 1 U AmpliTaq® Gold DNA polymerase, and 50 ng DNA template according to the following program: 10 min at 95°C; 45 cycles of 15 s at 95°C; 30 s at 60°C; and 30 s at 72°C with a final extension step of 7 min at 72°C. PCR products were analyzed on a 2% agarose gel and stained with ethidium bromide. A 100-base pair (bp) DNA ladder (Ecogen Biología Molecular, Barcelona, Spain) and *PstI*-digested phage lambda were used as a molecular weight marker. Long-range PCR reactions were performed with TCF AND 35SR primer (5 TCCTCTCCAAATGAAATGAACTTCC 3) and TaKaRa LA Taq polymerase (TaKaRa Bio Inc. Shiga, Japan) according to the instructions of the manufacturer.

TC-1507 QPCR Reactions

The Primer Express™ v. 2.3 software (Applied Biosystems) was used to design the oligonucleotides. The TaqMan® probe (Eurogentec, s.a., Seraing, Belgium) was labeled with the reporter dye fluorescent 6-carboxyfluorescein (FAM) on the 5 -end, and the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) was attached to its 3 -end. QPCRs were performed in an ABI Prism™ 7700 Sequence Detector System (Applied Biosystems) with SensiMix® core reagents (Quantace Ltd, Hertfordshire, UK) in a 20 L final volume containing 1× reaction buffer with internal reference, 6 mM $MgCl_2$, 300 nM TCF and TCR primers, 200 nM TCP probe (5 -FAM-ATCACAAACCGAGAGAAGAGGGATCTCGA-TAMRA-3), 10 l deoxinucleotide mix (Sensimix dU), 0.3 U uracyl-*N*-glycosylase (AmpErase UNG, Applied Biosystems), 1 U *Taq* polymerase, and 2 l template DNA at 50 ng/ L. Both event-specific target sequence and maize endogenous control *hmgA* were amplified in separate tubes under the following conditions: 2 min at 50°C (UNG-glycosylase activation); 10 min at 95°C; and 45 cycles of 15 s at 95°C and 1 min at 60°C. QPCR products were analyzed using the Sequence Detector System v.1.7 software (Applied Biosystems). Quantification was performed using the standard curve method. Threshold cycle (C_T) values of 45 were considered negative. All reactions were performed at least in triplicate.

For specificity tests, 100 ng genomic DNA extracted from all available CRMs corresponding to different maize transgenic events, as well as from other plant species, were used.

Sensitivity and Quantification Capacity

Genomic DNA isolated from powdered CRM 10% TC-1507 maize was serially diluted to final concentrations of 1000, 500, 100, 10, 4, 2 and 1 target molecules per 2 L. The copy numbers of the TC-1507 target sequence were calculated considering the size of the maize haploid genome, i.e., 2292-2716 bp (21), and the molecular weight of double-stranded DNA (965 Mb corresponds to 1 pg): approximately 2.6 pg genomic DNA corresponds to 1 maize haploid genome. QPCR and PCR reactions were performed in a total of 14 replicates along 3 independent experiments. The limit of detection (LOD) corresponded to the smallest number of target molecules per PCR that could be detected with 95% confidence. The relative LOD was assessed by analysis of TC-1507 CRM at 10, 1, 0.1, and 0%.

The linearity was assessed by R^2 values of the regression curve built with mean C_T values and initial numbers of target molecules. The efficiency of the reaction was calculated as $E = [10^{(-1/s)}]-1$, with s the slope of the same regression curve and 1 the optimal result. Acceptance criteria are $R^2 \geq 0.98$ and $-3.1 \geq s \geq -3.6$ (http://gmo-crl.jrc.it/guidancedocs.htm).

Validation Studies

A scheme was designed consisting of (i) 6 samples for standard curves and sensitivity assays corresponding to 10% TC-1507 CRM genomic DNA at the following concentrations: 1000, 500, 100, 10, 4, and 2 target copies/2 L; (ii) 3 unknown samples prepared from 10, 1, and 0.1% TC-1507 CRM, thus covering the dynamic range of 0.1–10% GM level; and (iii) maize wild-type genomic DNA (TC-1507 isogenic line CRM) as negative control. In addition, DNA-free samples were analyzed. All genomic DNA samples were produced by the developer laboratory. All samples were analyzed on 4 different PCR runs in each laboratory (i.e., developer and receiver); and each sample was triplicated in each PCR run. The *hmgA*-based assay (23) was used as a reference gene system to allow determination of the TC-1507 percentages in the unknown samples. Mean GMO percentage and relative standard deviation (RSD) values were calculated per each sample both in repeatability and reproducibility conditions. The percentage of bias was also calculated to estimate the trueness of the assay.

Results and Discussion

Characterization of 5 Flanking Sequences of Maize Event TC-1507; Design of Event-Specific QPCR and PCR Assays

Publicly available information regarding the introduced DNA in the maize event TC-1507 (www.agbios.com) indicates that the parental transgenic line incorporated one single copy of an intact fragment containing both the *cry1Fa2* and *pat* gene constructs with their associated non-coding regulatory regions, and a second copy of the *cry1Fa2* coding region lacking the majority of the associated ubiquitin regulatory sequences. Additionally, these genetic constructs were stably inherited and co-segregated over several generations of backcrossing. Therefore, we designed a TAIL-PCR strategy to amplify genomic sequences located 5 of the TC-1507 transgenic ubiquitin promoter on the basis of reported sequences (24).

A 1.3 kbp fragment was obtained with TC-1507 template DNA but not with the isogenic line. It was cloned and sequenced to further characterize this junction sequence (GenBank Acc. No. AM182233). As expected, its 3 sequence corresponded to the 5 portion of the *ubiZM1* promoter (i.e., homologous to nucleotides 2 to 298 of Acc. No. S94464). Beyond this ubiquitin promoter was a succession of short fragments with high homology to different available sequences (Figure 1). The proximal 63 bp sequence included a polylinker and might correspond to pHI8999 plasmid DNA used for transformation. Next, a 123 bp sequence included 43 bp of the same polylinker sequence and 80 bp of the same *ubiZM1* promoter. Further upstream was an unidentified 31 bp sequence and a 15 bp fragment sharing some homology with *cry1F*; after what were 2 sequences homologous to the *patII* gene frequently used in vectors (e.g., Acc. No. AY562548) and particularly in the pHI8999 insert (103 bp reverse and 201 bp forward); a 321 bp sequence corresponding to the

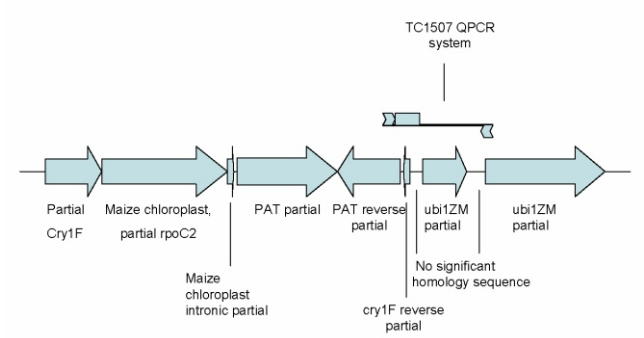

Figure 1. Schematic representation of the 5 TC-1507 transgenic map. Placement of primers and probe used for QPCR are represented above the map.

maize chloroplast *rpoC2* gene (Acc. No. X86563); and a 126 bp fragment with around 80% homology to the *B. thuringiensis cry1F* gene (Acc. No. M63897), probably corresponding to a part of the transformed modified *cry1F* gene (Figure 1). The European Food Safety Authority (EFSA) published an opinion of the Scientific Panel on Genetically Modified Organisms (GMO Panel) on TC-1507 maize (25) stating that the inserted fragment is flanked by several fragments originating from the recipient maize plant chloroplast and nuclear genome and from PHI8999 fragment used for transformation. In the same line is a technical report by Food Standards Australia New Zealand (FSANZ; 26) that mentions one partial copy of each *cry*1F and *pat* in the 5' region relative to the insertion event. Our results are in agreement with these reports and provide further sequence information. Genetic rearrangements are known to occur with high frequency at the site of insertion of novel DNA during plant transformation, particularly using particle acceleration techniques (15). The combination of sequences from different origins (both from maize and transgenic) could be explained as rearrangements occurring during the transformation process, and therefore this particular arrangement of sequences could be considered as TC-1507 event-specific. For that reason we selected a 190 bp fragment encompassing sequences from *pat* (reverse), *cry1F* (reverse), polylinker, and *ubiZM1* origins as target for an event-specific PCR assay. The position of the designed primers and probe are represented in Figure 1. Placement of the QPCR target sequences at 5 of the full-length TC-1507 insert was confirmed by long-range PCR amplification and partial sequencing of a 2.3 kbp fragment by TCF and 35SR, a reverse oligonucleotide designed to recognize CaMV 35S promoter sequences (i.e., the 5 element of the *pat* gene construct cloned 3 next to the *cry1F* construct in pHI8999 plasmid used for transformation); sequences homologous to *ubiZM1* promoter and first intron, *cry1F*, ORF25 terminator, and CaMV35S promoter were identified. QPCR assays were optimized according to protocol P/N 402823 Rev D (Applied Biosystems, 2003); whereas conventional PCRs were optimized for annealing temperature and primers and $MgCl_2$ concentrations. Optimal conditions are described in the *Materials and Methods* section.

Specificity of the Assays

The specificity of the PCR assays was experimentally tested using 100 ng genomic DNA from 10 different plant GMO events (i.e., 9 maize and 1 soybean lines) and 12 non-GM plant species, notably including the non-GM maize line isogenic to TC-1507 (Table 1). Both the conventional PCR and the QPCR assays unambiguously detected only genomic DNA from TC-1507. We also assessed the capacity of the assays to produce positive results with TC-1507 material from different sources. To that end, ground TC-1507 seeds provided by Pioneer were analyzed and positive results were obtained. Additionally, up to 10 different maize leaf samples from authorized field trials conducted in Spain were subjected to PCR and showed positive TC-1507 amplification. Therefore, the assays were considered appropriate for use in TC-1507-specific analyses of diverse types of samples.

Table 1. Specificity of TC-1507 QPCR and conventional PCR assays tested with 100 ng genomic DNA extracted from different plant GMO events and non-GM plant species.

	QPCR[a]	PCR
GMO events		
Maize event TC-1507		
Maize event 176	—	—
Maize event Bt11	—	—
Maize event NK603	—	—
Maize event GA21	—	—
Maize event CBH351	—	—
Maize event MON810	—	—
Maize event T25	—	—
Maize event Bt10	—	—
Soybean event GTS-40-3-2	—	—
Non-GM plant species		
Z. mays (maize):event TC-1507 isogenic line	—	—
S. bicolour (sorghum)	—	—
O. sativa (rice)	—	—
H. vulgare (barley)	—	—
B. napus (rapeseed)	—	—
B. oleracea (oilseed rape)	—	—
S. tuberosum (potato)	—	—
L. esculentum (tomato)	—	—
H. annus (sunflower)	—	—
T. aestivum (wheat)	—	—
A. thaliana	—	—
G. hirsutum (cotton)	—	—

[a] + = 15 Positive signals obtained in a total of 15 reactions;
— = 5 Negative signals obtained in a total of 5 reactions.

Table 2. QPCR values obtained with decreasing amounts of genomic DNA extracted from TC-1507 CRM[a]

Template molecules approximate	1000	500	100	10	4	2	1
Signal ratio (positive signal/total reactions)	14/14	14/14	14/14	14/14	13/14	9/14	7/14
Mean C_T values[b]	27.80	29.15	31.40	34.90	37.42	38.80	39.59
SD C_T valuesa[b]	0.25	0.21	0.21	0.50	2.43	2.95	2.09
RSD C_T values (%)[b]	0.90	0.72	0.67	1.43	6.49	7.60	5.28

[a] C_T refers to threshold cycle value, SD is the standard deviation, and RSD is the relative SD.
[b] Negative results were excluded from calculations.

Limits of Detection (LOD) and Quantification (LOQ)

The sensitivity of the developed QPCR assay was assessed by performing reactions (14 replicates along 3 different runs) on appropriate dilutions of TC-1507 genomic DNA to yield an estimated number of target molecules ranging from 10^3 to 1. As shown in Table 2, consistent detection was achieved down to 10 molecules; 4 target copies were detected with 93% probability and 1 single molecule could be detected in half of the replicates. This placed the absolute LOD (i.e., the lowest copy number that exhibits positive results in 95% of the replicates) between 10 and 4 target molecules. These results were consistent with statistical studies that considered the error associated with serial dilution processes and are in the range of other QPCR assays reported for other GMO events (5, 10, 27, 28). Our conventional PCR assay also achieved consistent detection of 10 target copies and 4, 2, and 1 target molecules in a percentage of the replicates (data not shown). We further tested the relative LOD of the assays by running triplicate reactions with 10, 5, 2, 1, 0.5, and 0.1% TC-1507 CRM. Positive results were detected in all reactions, thus further proving that the developed QPCR and PCR assays fulfill the LOD requirements of the EU legislation (i.e., 0.9% approved GMO is established as a threshold above which labeling is compulsory).

For QPCR, C_T values were plotted against the initial number of target molecules (10^3 to 10 molecules) to build a linear regression curve. It was highly linear ($R^2 = 0.99$), which demonstrated the quantification capacity of the system. The slope of the linear regression curve indicated a very efficient amplification rate (E = 0.95). Based on our experimental results and statistical considerations (22), the LOQ (i.e., the lowest copy number that exhibited linear correlation with the C_T, with R^2 values above 0.99 and nonoverlapping SD among contiguous DNA dilutions) was placed below 100 copies. These results are also in the range of other QPCR assays reported for other GMO events (10–12,16–18, 23, 29–34) and fit with the ENGL acceptance criteria.

Transferability of the TC-1507 QPCR Assay

Following the recommendations of the European Network for GMO Laboratories (http://gmo-crl.jrc.it/guidancedocs.htm) and according to the modular analytical procedure and validation approach (35), we tested the performance of the TC-1507 QPCR assay in a different laboratory. The experiment was intended as a preliminary test to evaluate the possible transferability of the developed QPCR assay. It consisted of the analysis of 4 unknown samples prepared from TC-1507 CRM (10, 1, 0.1, and 0%); triplicate reactions were run at each laboratory on 4 different days. In parallel, a series of serial dilutions of genomic DNA extracted from 10% TC-1507 CRM (from 10^3 to 2 target molecules per reaction) was also analyzed in order to corroborate the LOD and the efficiency of the QPCR. The assay displayed very similar performance values in the 2 laboratories, which were consistent with the ones defined above, i.e., positive detection of all tested samples down to 10 target molecules and

Table 3. Main results of the transferability assay[a]

Unknown sample TC-1507 %	10	1	0.1
Mean value developer laboratory (n = 12)	9.19	1.16	0.13
Mean value receiver laboratory (n = 12)	9.34	1.01	0.09
Mean value (L = 2)	9.27	1.09	0.11
Repeatability developer laboratory RSD (n = 12)[b]	15.71	10.15	18.93
Repeatability receiver laboratory RSD (n = 12)[b]	11.81	10.55	15.71
Reproducibility RSD (L = 2)[b]	11.29	10.92	20.14

[a] Unknown sample TC-1507% are values of the CRM used as unknowns. RSD is the relative standard deviation. All results are expressed in %.
[b] Repeatability and reproducibility estimates are for QPCR quantification and do not include extraction variation.

detection of 2 target molecules in a percentage of replicates, R^2 above 0.998, and E above 0.93. As expected, the endogenous control QPCR also performed adequately in the 2 laboratories.

The main results of the transferability trial are shown in Table 3. Its experimental design demonstrated that measured GM percentages were close to the true ones (i.e., bias <25%). Repeatability RSD values were <25%, and reproducibility RSD values were <30%, thus proved adequate for this technology (36). The results from the 2 different laboratories indicate the transferability of the assay; therefore, its validation in collaborative trials is recommended.

In conclusion, we characterized of 5 flanking sequences of maize event TC-1507 and unveiled rearrangements involving maize chloroplast and transgene DNA sequences in this genomic site. Such TC-1507 unique sequences were used as targets for event-specific QPCR and conventional PCR assays, which were fully specific and displayed LODs below 10 target copies. The QPCR assay was adequate for quantification of GM percentages, with adequate performance values. Finally, the capacity of the assay to be transferred to a receiver laboratory was established and, therefore, its validation in collaborative trials may be initiated.

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