

## Research Article

# Optimization of PCR DNA Sequencing Method for SNP Detection in Abacavir Sensitivity Gene

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- Nucleoside analogue Reverse Transcriptase Inhibitor (NRTI) drug
- HIV
- HLA-B\*5701
- Abacavir hypersensitivity reaction (AHR)

## Abstract

**Background:** Abacavir is an antiretroviral indicated in combination with other antiretroviral agents for the treatment of HIV-1 infection. It is associated with potentially serious hypersensitivity reactions in 3–9% of Caucasian patients. Carriers of *HLA-B\*5701* are at high risk for developing abacavir hypersensitivity reaction (AHR). In Caucasians, *HCP5 rs 2395029(G)* is in high but not complete linkage disequilibrium (LD) with *HLA-B\*5701*.

**Aim:** To development of fast method to screening of *HLA-B\*5701* allele, by optimization of PCR DNA Sequencing method for SNP detection in Abacavir Sensitivity gene.

**Methods:** DNA extraction by whole blood Sample using the QIAamp® DNA Blood mini kit (Qiagen, Hilden, Germany). *HLA-B\*5701* allele Specific amplification Primer is design in Primer3 ( online tool ) using db SNP ID-rs2395029 as Reference Sequences, these Primer is used PCR and PCR DNA Sequencing . SNP detected by Chromas Software using db SNP ID-rs2395029 as Reference Sequences.

**Results:** We propose a simple approach to detection of SNP of *HLA-B\*5701* allele associated abacavir hypersensitivity based on optimization of PCR DNA Sequencing Method of *HLA-B\*5701* allele to determine the presence of hypersensitivity risk.

**Conclusion:** *HLA-B\*5701* genotyping before initiation of therapy remain probative of .the Introduction of highly active antiretroviral therapy has Transformed the nature of HIV Infection from a severe and ultimately fatal disease to that of a manageable chronic condition. HIV drugs are highly efficacious, but there use comes at the cost of a range of drug –related adverse events, including sever hypersensitivity reaction (HSRs) that have been most notably associated with abacavir and other NRTIs drug therapy.

## ABBREVIATIONS

PCR: Polymerase chain reaction; AHR: Abacavir hypersensitivity reaction; HLA: Human leukocyte antigen; HSRs: Hypersensitivity reaction; NRTI: Nucleoside analogue reverse transcriptase inhibitor; MHC: *Major histocompatibility complex*; ABC-HSR: Abacavir hypersensitivity reaction; EDTA: Ethylenediaminetetraacetic acid; OD: Optical density; SNP: *Single Nucleotide Polymorphism*

## INTRODUCTION

Abacavir is a potent nucleoside analogue reverse transcriptase

inhibitor (NRTI) drug that has been prescribed for the treatment of HIV infection since 1999, as a component of combination treatment regimens that typically include two NRTI drugs and a third drug from a different antiretroviral drug class (e.g. HIV protease inhibitors, non-NRTIs). Abacavir is currently available and used either as a single drug (Ziagen™) or in coformulated drugs that combine two NRTI drugs (abacavir/lamivudine: Epzicom/ Kivexa™; abacavir/zidovudine: Combivir™) or three NRTIs(abacavir/lamivudine/zidovudine:Trizivir™) [1]. It is generally a very well-tolerated drug, even though an increased risk of myocardial infarction in patients exposed to ABC within the preceding 6 months [2], but not confirmed by other studies

[3]. The major reason for early discontinuation of ABC remains the occurrence of an immunologically mediated hypersensitivity reaction (HSR) [4].

ABC-HSR has been observed in 5–8% of treated patients, appears within the first 6 weeks of therapy and requires an immediate and permanent discontinuation of the drug [5]. A strong association between the ABC-HSR and MHC class I allele *HLA-B\*57:01* (locus position 6p21.3 and OMIM: 142830) has been observed in Caucasian HIV-infected patients [6,7].

Subsequently, skin patch testing demonstrated the presence of such an association across racial and geographic populations [8,9]. Finally, a powered, double-blind, prospective, randomized study (Prospective Randomized Evaluation of DNA Screening in a Clinical Trial [PREDICT-1]) demonstrated the clinical utility of *HLA-B\*57:01* screening in reducing ABC-HSR in HIV-infected patients. HSR was clinically diagnosed in 93 patients from a total of 1650 patients, with a significantly lower incidence (3.4%) in the prospective screening group than in the control group (7.8%;  $p < 0.001$ ). Interestingly, *HLA-B\*57:01* screening eliminated immunologically confirmed HSR, with a negative predictive value of 100% [9]. Therefore, international HIV treatment guidelines now recommend *HLA-B\*57:01* typing before ABC administration, in order to reduce the incidence of ABC hypersensitivity [10,11,11]; while the screening is also part of the license of ABC-containing products.

By PCR and Sanger sequencing Method used to optimized the *HLA-B\*5701* allele in Patient DNA samples with sequence specific *HLA-B\*5701* forward and reverse Primer, to detected by SNP in Chromas bioinformatics Software .Our finding Suggest that detection of SNP-genotyping is Confirmation method of the Hypersensitivity will be favor to the Patient's Safety.

## MATERIAL & METHODS

### DNA Sample

A 12 blood sample was provided by the geneOmbio Technologies Pvt Ltd. ,Baner, Pune , Maharashtra (India). These patient samples send to geneOmbio Technologies Pvt Ltd. To diagnose the HIV infection in different clinic and Hospital in India. Whole blood samples were collected in EDTA treated tubes and used in present study. These sample initially used to optimize the experimental conditions, as detailed below. These sample came from indian ethnic population and were genotyped by sequence-specific by low-resolution sequence-specific oligonucleotide PCR , followed by high -resolution sequence specific oligonucleotide PCR carried out on the *HLA-B\*5701* positive samples (geneOmbio Technologies Pvt Ltd. , Baner, Pune, Maharashtra -India ).

The latter method was validated during the PREDICT-1 clinical trial on 1956 samples [9], by comparison with the gold standard technique based on DNA sequencing.

### Total DNA extraction

Total DNA was prepared from whole blood samples, using the QIAamp® DNA Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, whole blood was collected in EDTA-treated tubes and stored at -20°C until the day of the extraction. Aliquots of 200 µl for each whole

blood sample were used, and total DNA was eluted in 200 µl elution buffer (10 mMTrisHCl; 0.5 mM EDTA; pH 9.0; provided by the kit). DNA concentration was measured by using UV Spectrophotometric Analysis. The DNA sample is calculated as following Method (Table 1).

The absorbance of a DNA sample at 280 nm (OD) gives an estimate of the protein contamination of the sample. The ratio of the absorbance at 260 nm/absorbance at 280 nm is a measure of the purity of a DNA sample; it should be between 1.65 and 1.85. final DNA yield was approximately 5 µg per sample (Figure 1).

### HLA-B\*5701 allele-specific amplification by PCR

*HLA-B\*5701*-specific amplification primer is design in Primer3 (online Tool) using db SNP ID - rs2395029 as Reference Sequences, for PCR using the following validated primers: *HLA-B\*5701* forward primer (forward: 5'-CAGGAACTCCTCCTACCCTC-3'), and *HLA-B\*5701* reverse primer (reverse: 5'-GGACCCACGTGTTCTTCCCTA -3'), yielding a 341 bp product. The PCR reaction contained 5.0 µl total DNA, 1X PCR buffer 2.5 µl , MgCl 0.75 µl , dNTPs 0.5 µl, Tag Polymerase (Invitrogen Corporation) 0.2 µl, *HLA-B\*5701* each forward and reverse Primer 1.0 µl in 25 µl final volume. After initial denaturation (5 min at 95°C), a total of 35 PCR cycles were conducted, using the following two-step PCR conditions: denaturation at 94°C for 30 min and annealing at 55°C for 45 min /extension at 72°C for 45 min, and final extension at 72°C for 10 min in a 2720 thermal cycler Life technologies (AB 2720 model). The amplicons were separated by electrophoresis through 2% agarose gels containing 0.1 µg/ml ethidium bromide. This step allowed us to verify the efficiency of DNA amplification, in order to avoid the processing of incorrect pre-amplified samples, thus reducing the incidence of false-negative results.

*HLA-B\*5701* allele was amplified and it shows the intact band on Gel, and compared with ladder sequence of 100-500 bp.

**Table 1:** DNA concentration was measured by using UV Spectrophotometric Analysis of the DNA sample.

Patients Sample No.	A260 (OD)	A280 (OD)	Protein (Contaminated) Concentration (mg/ml)	
			(A260/A280)	(A260/A280)
DNA Control ( NTC )	0.19	0.11	0.0261	1.73
P1	0.35	0.31	0.2145	1.12
P2	0.32	0.30	0.2217	1.06
P3	0.34	0.36	0.2995	0.94
P4	0.38	0.32	0.2072	1.18
P5	0.31	0.34	0.2914	0.91
P6	0.34	0.36	0.2995	0.94
P7	0.30	0.33	0.2835	0.90
P8	0.35	0.32	0.2300	1.09
P9	0.32	0.31	0.2372	1.03
P10	0.37	0.35	0.2613	1.05
P11	0.33	0.36	0.3071	0.91
P12	0.36	0.38	0.3154	0.94

The expected PCR product size 341 bp can be estimated from the gel electrophoresis image of Standard PCR product.

### Purification of PCR products using PureLink® PCR Purification Kit (Invitrogen by life technologies)

Use the PureLink® PCR Purification Kit to efficiently remove primers, dNTPs, enzymes, and salts from PCR products in less than 15 minutes. Use the kit with Binding Buffer High-Cutoff (B3) to remove primer dimers or short spurious PCR products (Figure 2).

The PureLink® PCR Purification Kit is based on the selective binding of dsDNA to silica-based membrane in the presence of chaotropic salts. Mix a PCR product with Binding Buffer to adjust conditions for subsequent dsDNA binding to the PureLink® Spin Column. The dsDNA binds to the silica-based membrane in the column. Remove impurities by thorough washing with Wash Buffer. Purify the DNA, elute the dsDNA in low salt Elution Buffer or water. The purified PCR product is suitable for automated fluorescent DNA sequencing.

### HLA - B\*5701 genotyping by PCR, DNA Sequencing for SNP Detection

HLA-B\*5701 genotyping was performed by DNA sequencing using allele specific PCR primer with BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technology) and the genetic analyzer 3130 X L (Applied Biosystems, CA, USA). Sequence analysis was carried out Chromas Lite Software (RASA Life Science Informatics, Pune - India).

To genotype HCP5 rs2395029, we prepared Master Mix for PCR DNA Sequencing. the Master Mix contained 3.0 µl Patient



**Figure 1** 1% Agarose (w/v) Gel Electrophoresis : Isolated DNA Product in Human Blood Sample P1 – P12; And Control – NTC .



**Figure 2** 2% Agarose (w/v) Gel Electrophoresis of HLA-B\*5701 allele PCR Purification Products for Sequencing of Patient DNA Sample: Ladder : 500 bp DNA Size standard ; Lane 1 to Lane 12 – Patients DNA PCR sample and NTC –control .

DNA Sample, Allele Specific Design Primer :HLA-B\*5701 forward primer (forward: 5'-CACGAACCTCCTACCCTC -3') is 1.0 µl, Ready Reaction Mixes (R.R. Mix) 0.5 µl, Sequencing Buffer 1.8 µl SMQ 3.7 µl. We amplified a 341-bp fragment containing the rs2395029 SNP.

For Sequencing PCR cycles were conducted, using the following step PCR conditions: Initial denaturation at 96°C for 1 min, denaturation at 96°C for 10 Sec, annealing at 50°C for 5 Second extension at 60°C for 4 min, and final Cooled at 4°C for ∞ in a 2720 thermal cycler Life technologies (AB 2720 model). Amplicons were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified by BigDyeXTerminator® (Applied Biosystems) prior to loading on the ABI 3730×L DNA Analyzer. ABI files were analyzed with Chromas Software for sequence analysis.

## RESULTS

### PCR optimization of HLA-B\*5701 allele of 12 Patients DNA Sample

A fragment of size of 341 bp is expected to be generated that is specific to HLA-B\*5701 when amplified using primer pairs: HLA-B\*5701 forward primer (forward: 5'-CACGAACCTCCTACCCTC -3'), and HLA-B\*5701 reverse primer (reverse: 5'-GGACCCACGT-GTTCTCCTA -3') respectively. Approximately 200 ng of sample DNA (Standard Human DNA) was used for PCR amplification.

The PCR amplification reaction mix of 60 µl contained sample DNA, 2.4 µl Taq-DNA polymerase, 2.5 µl of 10x PCR buffer, 0.5 µl of 10 mM dNTP mix and 1 µl of each primer (10 pMol/µl). Amplification was carried out using a GeneAmp PCR System (Applied Biosystems, USA). Sterile nuclease free water was used in place of DNA as a negative control. The amplified DNA fragments were resolved on a 2% agarose gel spiked with Ethidium

bromide. The gel was visualised under UV transilluminator and photographed using GelDoc XR documentation system (BIO-Rad).

Thermal cycling conditions optimized for this amplification were: 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 55°C for 45 sec and 72°C for 45 min; final extension was performed at 72°C for 10 min and final hold at 4°C.

Results of optimization PCR indicated that amplification using primer HLA-B\*5701 forward primer ( forward: 5'-CACGAACTC-CTCCTACCCTC-3'), and HLA-B\*5701 reverse primer (reverse: 5'-GGACCCACGTGTTCTTCCTA -3') is giving highly amplified PCR products .hence the primer HLA-B\*5701 forward primer (forward: 5'-CACGAACTCCTCCTACCCTC -3'), and HLA-B\*5701 reverse primer ( reverse: 5'-GGACCCACGTGTTCTTCCTA -3') was chosen for Sequence Analysis for SNP Detection with study samples (Figure 3).

### Sequence Analysis for SNP Detection

PCR product obtained from standard human genomic

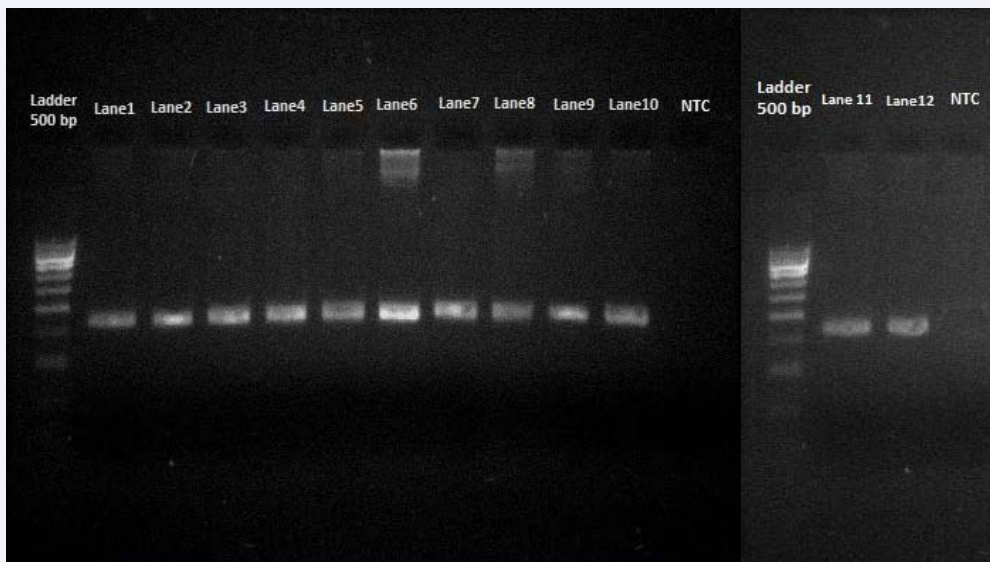
DNA was purified using geneOmbio Pure PCR purification kit to remove unused dNTPs, primers and reagents from the amplification product. This product was further subjected DNA sequencing using Applied Biosystems BigDye Terminator v3.1 Cycle sequencing kit on Automated DNA sequencing platform GA3130 and SNP detected by Chromas Bioinformatics Software by using Reference Sequences (db SNP ID - rs2395029 ) (Figure 4).

### Mutation analysis

Sequence analysis showed that single nucleotide polymorphism exists only at position rs2395029 in the completed sequences. The polymorphic sequences were either mixture of G + T or only G and T at this specific position (Table 2).

### Patient DNA Sequence Chromatogram indicating mutation positions

The output of sequence in Chromas Software shows excellent chromatogram, with clean, distinct and very low to no back ground noise. The Sequence of HLA-B\*5701 allele for HLA-



**Figure 3** 2%Agarose (w/v) Gel Electrophoresis of HLA-B\*5701 allele PCR Products: Ladder: 500 bp DNA Size standard; Lane 1 to Lane 12 – Patients DNA PCR sample and NTC –control.

```
>RefHLAB5701
GCGACTGAAATCTTCCTTCCTGAGGGGTCTGGGCTCTTGGAAATCAAACCTCTCAGGTTGGGTGGCTG
GACGATTCTCTCACACTTACAATGGGACAAGGGGAACCAAGGAGGCCCAAGGGGATCCCTGGGTTC
CACACGAACTCCTCCTACCCTCATTGTGTGACAGCAGCCATGCCTCCTCCTGGGGATCAGGATCTATTA
CCTGTGCCTGGAGAGGAGGGGACTCCTTCTCACCCGCTGGTCTCTGGACACATACTGTCCAATTCCC
CTGKGGCAGCTGTAATGTGTAGTTCAATGGGCACTCATTTGTCCCTTTTAAAGGGTACCCTCCTTTAGA
ATCCAGGACCTTCTACCCTGCAGAGTGTGGTTTTGGGAGAGAAGTGCAAAATCCCACGACAGGTGAGT
TGAAGGAATGGGATATGGAGCCACATCCACTTCCACCCCTTGGTATCTGGACCCACGTGTTCTTCCTAC
TGAGATTACAGAAGTGTAGAGATGCTTTGATTTTTAAAATGCACCATGTCCTGAAAGATGGCACCCCTC
CCACCGA
```

**Figure 4** The Reference Sequences is given from using db SNP ID - rs2395029.

B\*5701(F) Primer. It shows the general appearance of the chromatogram peaks (Figure 5).

**DISCUSSION**

In the paper we have validated a rapid pharmacogenomic test for the screening of the *HLA-B\*57:01* allele using designing *HLA-B\*5701*-specific amplification primer in Primer3 (online Tool) using db SNP ID - rs2395029 as Reference Sequences (approximately 20 bp Oligonucleotides and product size 341 bp ) that aligned the *HLA-B\*57:01* sequence in two different regions. PCR Optimization and PCR DNA Sequencing of *HLA-B\*5701* Allele. Since the *HLA-B\*57:01* sequence analyzed from the 12 study samples. However it was found that the rs2395029 SNP existed either as TT (Homozygous normal) or GT (Heterozygous mutant) only (Table 2 and 3).

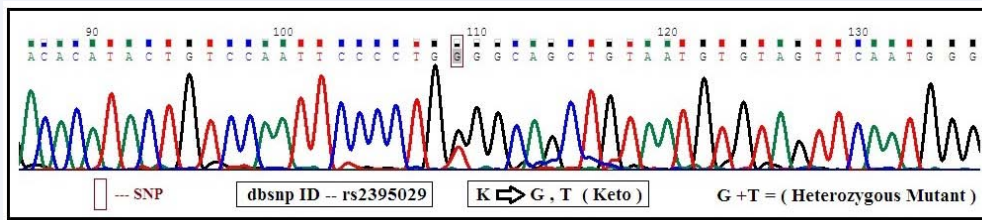
In 12 samples were for testing and were used to characterize the prevalence of *HLA-B\*5701* and *HCP5* rs2395029. These samples were successfully genotyped for *HLA-B\*5701*. We found 1 individuals who were homozygote and 11 individuals who were heterozygote carriers of *HLA-B\*5701*, were heterozygote carriers of the G allele and homozygote also carriers T allele of rs2395029 (Table 3). There was complete agreement between the rs2395029 genotype by allelic discrimination and the genotype retrieved from the MGD database.

Following the identification of *HLA-B\*5701* as a clinically useful marker of susceptibility to abacavir hypersensitivity,

particular consideration has been given to the fact that HLA typing is generally a highly specialised and often expensive diagnostic technique that is offered in a limited number of laboratories involved in transplantation medicine. Hence, there has been an increased focus on developing accurate, robust and cost-effective methods for identifying the *HLA-B\*5701* allele that may be performed in HIV laboratories in a timely manner. These include the molecular HLA typing methods described earlier, which enable targeted assessment of the *HLA-B\*5701* allele [12]. Further refinements of these techniques could also provide for use of whole blood samples without requiring DNA extraction (as already demonstrated in the case of *HLA-B\*27* testing in the diagnosis of ankylosing spondylitis [13]). In the case of abacavir hypersensitivity, the favoured pathogenic model involves HLA-specific presentation of a peptide epitope that has been rendered antigenic by an abacavir metabolite (*i.e.* the 'hapten' model), within an immune environment that may also be shaped by genetic factors that are coinherited along with *HLA-B\*5701* on the 57.1 ancestral haplotype. It also seems likely that HLApeptide interactions specifically involve HLA residues located at the base of the peptide-binding groove such as the serine-116 position, again favouring a model of peptide antigen processing and HLA-restricted presentation rather than direct MHC drug binding on the cell surface [14].

**CONCLUSION**

In this paper, we provided evidence to suggest the adoption



**Figure 5** Output of Sequence in Chromas Software shows excellent chromatogram of P2 Sample *HLA-B\*5701* allele. It shows the Mutation Position of the chromatogram peaks.

**Table 2:** Mutation analysis Results of Patients Sample.

Patients Sample No.	Mutation found at Position (dbSNP ID)	SNP	Clinical Prediction of Abacavir Hypersensitivity
P1	rs2395029	T (Homozygous normal)	No Risk
P2	rs2395029	G+T (Heterozygous mutant)	Moderate Risk
P3	rs2395029	T (Homozygous normal)	No Risk
P4	rs2395029	T (Homozygous normal)	No Risk
P5	rs2395029	T (Homozygous normal)	No Risk
P6	rs2395029	T (Homozygous normal)	No Risk
P7	rs2395029	T (Homozygous normal)	No Risk
P8	rs2395029	T (Homozygous normal)	No Risk
P9	rs2395029	T (Homozygous normal)	No Risk
P10	rs2395029	T (Homozygous normal)	No Risk
P11	rs2395029	T (Homozygous normal)	No Risk
P12	rs2395029	T (Homozygous normal)	No Risk

**Table 3:** Clinical interpretation of HLA-B\*5701 Hypersensitivity tests.  
**Interpretation: Number of Samples showing polymorphism.**

SNP	Clinical Result	
GG	High Risk of Abacavir Hypersensitivity	
GT	Moderate Risk of Abacavir Hypersensitivity	
TT	No Risk of Abacavir Hypersensitivity	
	T (Homozygous normal)	G+T (Heterozygous mutant)
Patients (n=12)	11	1

of the test herein characterized into clinical practice as a fast method to screen for *HLA-B\*57:01* status, particularly in those laboratories already involved in detection abacavir Hypersensitivity Reaction and *HLA-B\*5701* genotyping.

The Test described may be considered as DNA Isolation, *HLA-B\*5701*-specific amplification primer is design in Primer3 (online Tool) using db SNP ID - rs2395029 as Reference Sequences. PCR Optimization of *HLA-B\*5701* Allele.

DNA sequence analysis of *HLA-B\*5701* allele revealed that apart from the reported mutation site at n.733T>G (rs2395029) mutation exists in the sequences analyzed from the 12 study samples. However it was found that the rs2395029 SNP existed either as TT (Homozygous normal) or GT (Heterozygous mutant) only.

Third form of the allele as GG was not found in these samples. The study illustrates that polymerase chain reaction and DNA sequencing technology can be used to study the SNPs from *HLA-B\*5701* allele using the primers designed and PCR Optimized here in. The genotype results can be provided in 24 h (30 patients) versus 48 h of the reference method (ten patients). The entire assay is run in approximately 6–8 h.

## FUTURE PERSPECTIVE

A SNP genotyping is a confirmation method will be favor the implementation of pharmacogenetic programs through accessible and affordable tests that will increase patient's safety. Pharmacogenetic tests will be increasingly available at clinical laboratories and will enable the practice of personalized medicine.

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## WEBSITE

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