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High Resolution Melting Curve Analysis of DNA Sequence Alterations of Various Sizes

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Abstract In the diagnostic workflow we need to think in algorithms, containing more assays. One of the most important task in the management of cancer patient is to detect nucleic acid sequence changes in clinical specimens. Before using the most expensive method to analyze direct our targets, a screening assay is needed to reduce the number of samples. In the detection of gene-sequence alterations classical screening methods are available, as SSCP, DGGE or TGGE, (Finke Exp Clin Endocrinol Diabetes 104:92–97, 1996; Lessa and Applebaum Mol Ecol 2:119–129, 1993) however these are very time consuming processes. At this time in the molecular lab the real-time PCR equipments are very popular and with the function of melting curve analysis it can be a very convenient, simple and cost-efficient screening method.

Keywords Melting curve · High resolution · DNA · Mutations · GIST

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Introduction

The aim of this study was to present a very fast and simple real-time PCR based screening test for the determination of positive samples with sequence alteration, to be selected for further analysis with direct mutation detection methods. With the use of saturating double strand specific dye [3] it is possible to identify one base alteration in a range of 100 to 160 bases PCR fragment. With this assay the polymorphic samples will be also filtered, with all of the possible alterations. After this screening, a probe [4–8] or sequencing based assays can be used for the determination of the concrete type of sequence variation.

In order to introduce this Strategy the analyzed target was the exon 11 sequence of the c-kit oncogene (4q11–q12); a tyrosine-kinase receptor, which is frequently altered in various human tumors, especially in GIST (Gastrointestinal Stromal Tumor) [9] Fig. 1.

Materials and Methods

Nucleic Acid Isolation and Purification with Magnetic Method

DNA was isolated from GIST formalin fixed and paraffin embedded (FFPE) samples of GIST from the pathological archive.

With magnetic beads isolation method the nucleic acid (NA) meets the capture surface with higher probability than with static surface, like in the case of a column based system. This “mobile surface NA capturing isolation” get more representative NA from the fragmented samples, because during the binding period of the purification, the silica coated magnetic particles are well suspended with the

Fig. 1 Human c-kit exon 11 sequence

>ref|NC_000004.10|NC_000004:55288339-55288465 Homo sapiens chromosome 4, reference assembly, complete sequence, exon 11 is 127bp long
AAA CCC ATG TAT GAA GTA CAG TGG AAG GTT GTT GAG GAG ATA AAT GGA AAC AAT TAT GTT TAC ATA GAC CCA ACA CAA CTT CCT TAT GAT CAC AAA TGG GAG TTT CCC AGA AAC AGG CTG AGT TTT G

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1741 ACAGAAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATAAATGGAAACAATTA
 548 --Q--K--P--M--Y--E--V--Q--W--K--V--V--E--E--I--N--G--N--N--Y

1801 TGTTTACATAGACCCAACACAACTTCCTTATGATCACAAATGGGAGTTTCCCAGAAACAG
 568 --V--Y--I--D--P--T--Q--L--P--Y--D--H--K--W--E--F--P--R--N--R

1861 GCTGAGTTTTGGGAAAACCCTGGGTGCTGGAGCTTTCGGGAAGGTTGTTGAGGCAACTGC
 588 --L--S--F--G--K--T--L--G--A--G--A--F--G--K--V--V--E--A--T--A
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sample. We used the Roche Magna Pure LC system with the combination of the MagnaPure Total NA kit. The sample volume and the elution volume was 1,000 µl deparaffinated sample in lysis buffer and 50 µl elute in PCR grade, nuclease free water.

For any PCR based analysis it is recommended to use a method to detect the quality of DNA samples (whether there are amplifiable templates). The sample quality was determined with real-time PCR testing. This control reaction detects the level of DNA degradation and the presence of PCR inhibitors. For this purpose a separate PCR control reaction was used. The beta globin gene specific amplification analyze the ability of PCR [10]. Next, the obtained Cp values can be converted to concentration with the use of calibration curve based on the parameters of known standard samples. This value is more useful than absorbance measurement with the combination of genomic DNA electrophoresis and smear detection, because this value indicates the amount of functioning DNA in a PCR reaction. In addition, the absorbance measurement was also performed, but only for analyzing the purity of the samples.

Designing the High Resolution Melting Real-Time PCR Assay for the Detection of c-Kit Oncogene Deletions

Target Selection

For the appropriate design we aligned more than 10 wild type sequences to eliminate the risk of false selection of the primer binding region. After the creation of consensus wild type sequence the deleted versions of c-Kit exons were aligned (Fig. 2). In this step the constant sequence regions can be located for designing the primer binding sites. For the well working HRM analysis, the short amplicons in the range from 80 to 160 bp are ideal [11]. Our fragment sizes were higher, because the intronic location of constant region for forward primer design. The sizes were than between 135 bp and 171 bp depending on the deletion size.

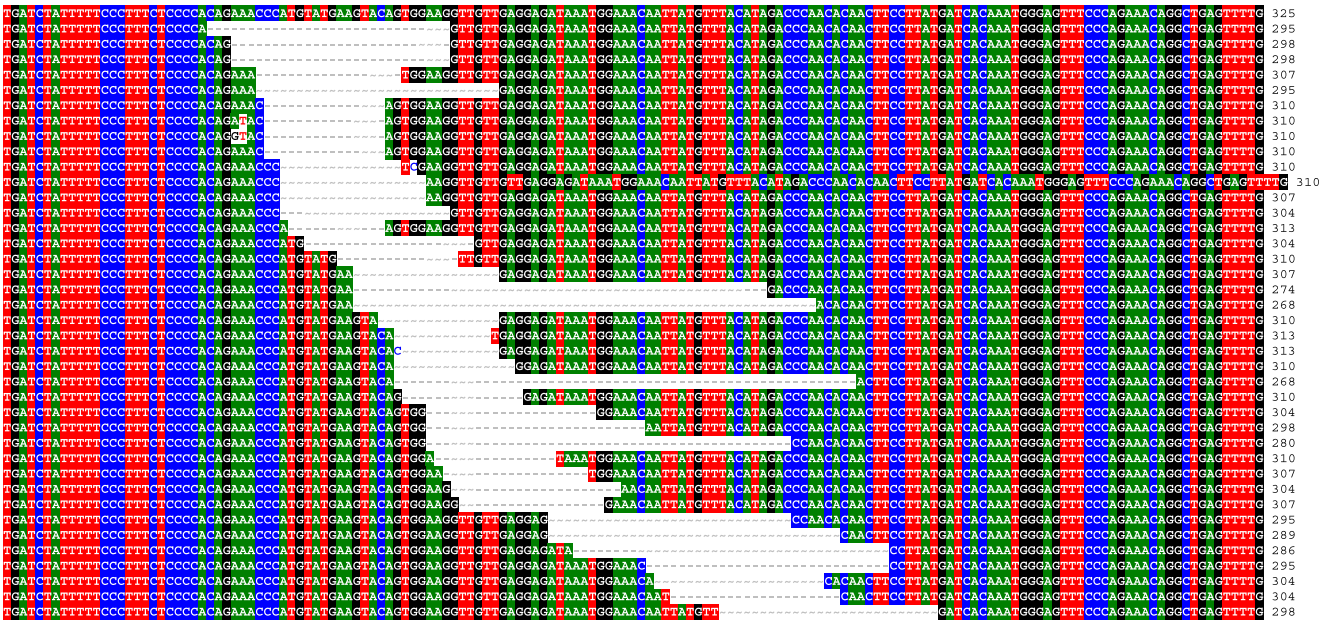

Fig. 2 Aligment of common c-kit exon 11 deletions

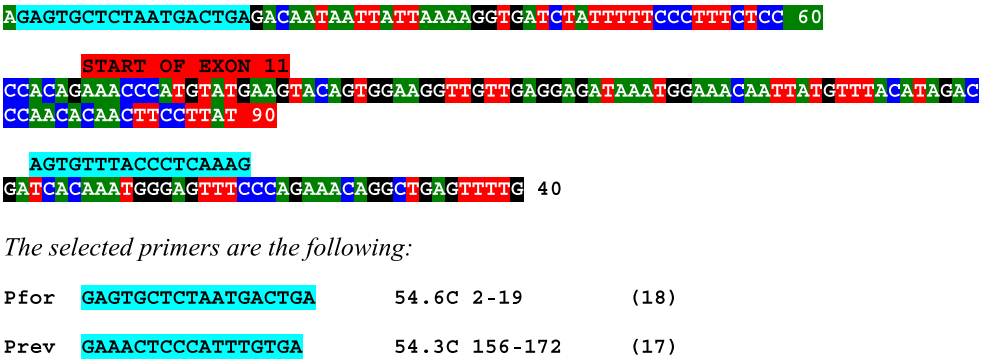

Fig. 3 Amplified fragment of c-kit exon 11 and the selected primers

The melting characteristic was predicted in-silico with the analysis tool on website: http://www.biophys.uni-duesseldorf.de/local/POLAND/, [12].

Aligned Sequence Variants

The frequently occurred sequence alteration can be found from motif CTCCCCA in intron, to GATCACAAA. That means, we can easily design the forward primer into the intronic region to avoid the amplification of non genomic fragments.

Primer Design

For this purpose we used 2 distinct softwares with the combination of hairpin detection algorithm, e.g. cyber gene, (http://www.cybergene.se/) . These programs are the Roche Probe design software 2.0. and Primer 3, (http://frodo.wi.mit.edu/primer3/).

With probe design software the selected region can be analyzed. Using the option of fixed oligos or performing regional search, we can position the primer sequences and fix the amplicon size. Now, the score of the designed primers and the hairpin possibilities have to be checked with a different algorithm, e.g: cybergene. For the successful optimalization it is strongly recommended to order more primer pairs to test more combinations, to get a dimer peak free melting shape. False shape can be present if we have high dimer melting profile in the temperature range of amplicon melting closely connected to the analyzed curve. It is also very important to use HPLC grade oligos to avoid the false binding and the formation of not predictable dimers with altered sequence containing primers. The primers were ordered from Tib-molbiol Gmbh, Germany (Fig. 3).

Optimalization of the Assay

For choosing the ideal conditions, standard human genomic DNA from the Roche DNA control kit was used to exclude the effect of influencing factors originated from the clinical samples and the isolation procedure. The reaction mixture was made up using HRM master (Roche Diagnostic GmbH, Mannheim), which also includes the saturating dsDNA specific dye. The $MgCl_2$ concentration was optimized between 1.5 mM and 6 mM concentration with a step size of 0.5 mM. Next, the concentration of the primers was defined, because the lowest, but well working value are needed. The titration range was between 3 to 10 pmol / 20 μl reaction volume. During setting this values we need to analyze the melting peaks and the amplification curves. We choose the values without additional dimer or mis-

Table 1 PCR melting conditions

Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
pre denaturation							
95	None	0:10:00	4.4	5	0	0	0
amplification							
95	None	0:00:10	4.4	5	0	0	0
63	None	0:00:10	2.2	5	55	1	10
72	Single	0:00:10	4.4	5	0	0	0
melting analysis							
95	None	0:00:30	4.4	5	0	0	0
40	None	0:01:00	2.2	5	0	0	0
60	None	0:00:01	1	5	0	0	0
95	Continuous	0:00:00	0.04	25	0	0	0

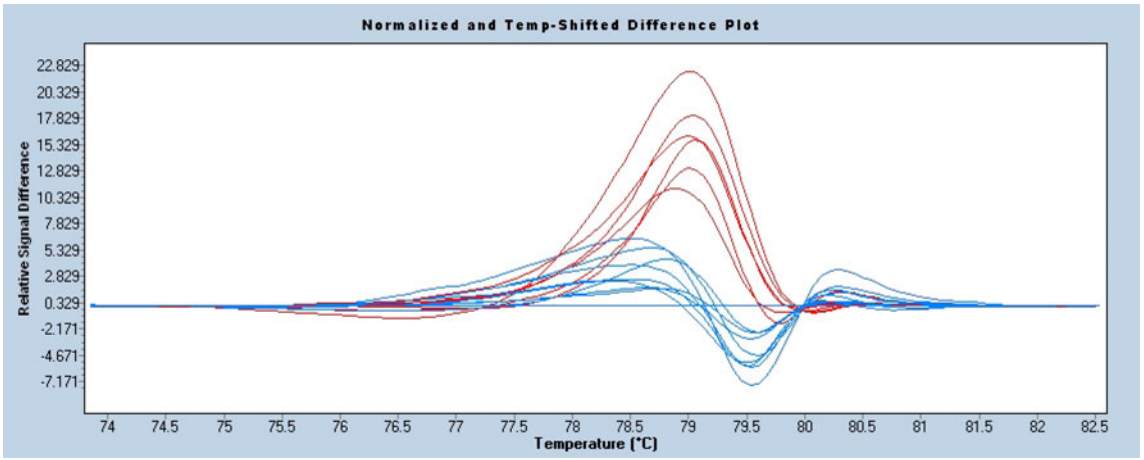

Fig. 4 Difference plot of wildtype (*blue*) and SNP sequences

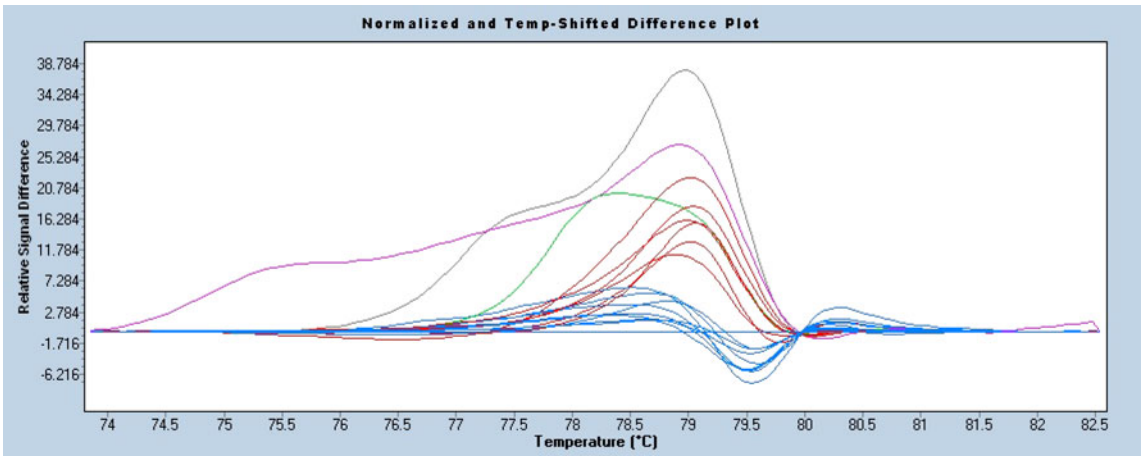

Fig. 5 SNP samples with deleted samples varying in deletion size

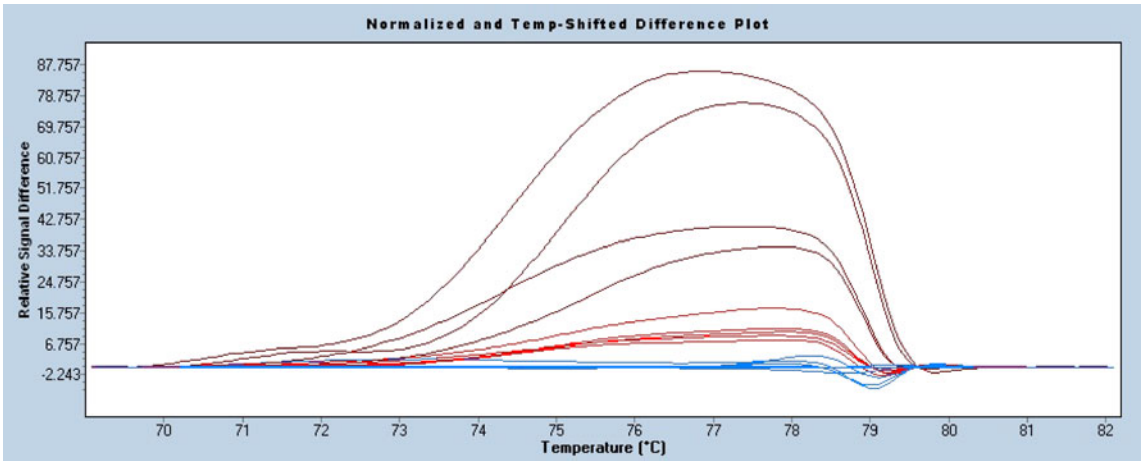

Fig. 6 Difference plot of long deletions over 6–8 basis, in various concentrated samples

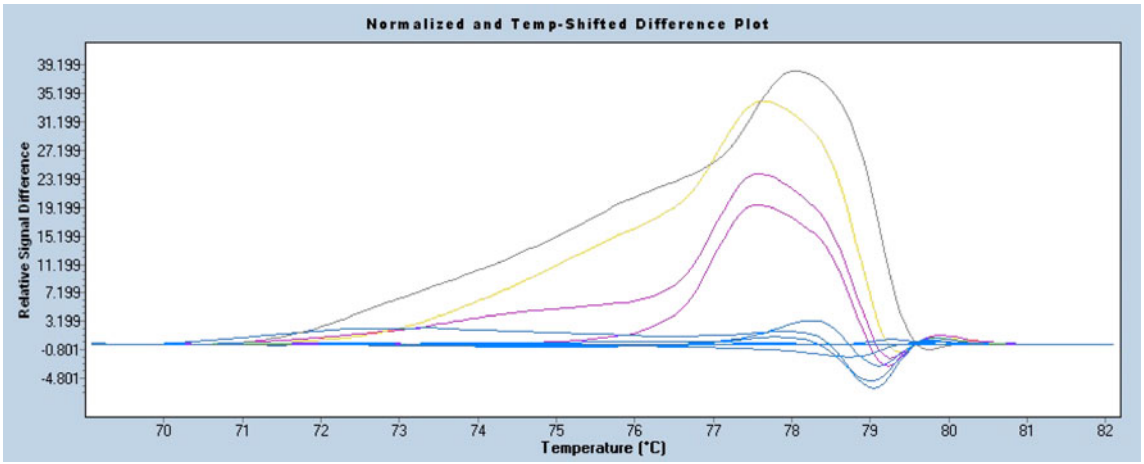

Fig. 7 Another type of shorter deletions with wild-type samples

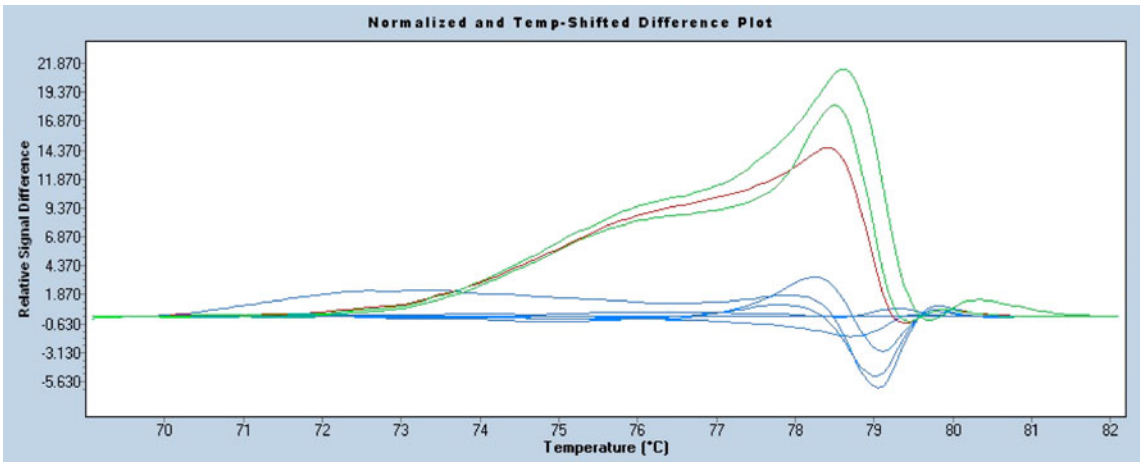

Fig. 8 Deletions with SNPs in heteroduplex

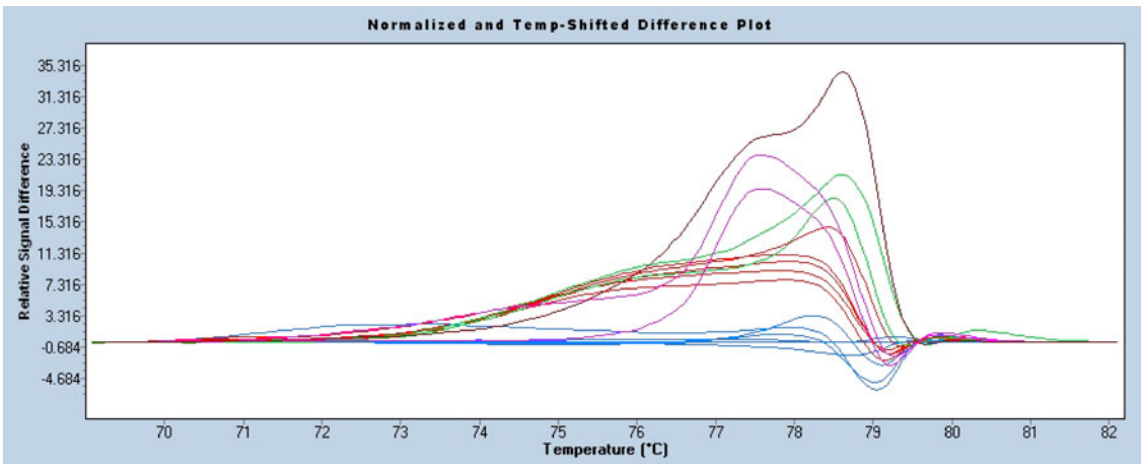

Fig. 9 All sample types together

product peaks, and with the highest exponential ramp rate during amplification.

For optimizing the annealing temperature touchdown PCR was used, what is often the best option for HRM PCR [13]. In this case, during the 5 to 10 first cycles we adjust a higher annealing temperature (63°C) to amplify and highlight the specific fragment from the genomic background. After the initial, specific but less effective amplification the annealing (55°C) temperature was lowered, to increase the effectiveness of the reaction. PCR cycling and HRM analysis was performed on the Roche LightCycler 480 system (Roche Diagnostics GmbH). The amplicons were run according to the following conditions (Table 1).

Melting analysis will provide characteristic melting curves. With a special curve manipulation the different curves can be aligned to detect informative alteration between the shapes. First the negative samples were filtered out for baseline correction. Next is to compensate the different start and end levels with baseline normalization. Which means a common start and end baseline for all sample curves. Then the temperature was shifted, what corrects the different ion levels, what can influence the melting point. After this corrections the shapes can be used for alignment.

The aim of our assay is to filter out the altered sequences from the wild-types. To achieve this, a consensus wild-type curve shape and many different shape types for distinct deletions and alterations were made.

Results

After using different control samples, the main alteration types in the sequence were identified. In the last software module the reference shape was fixed to serve as a reference curve. In any case with the wild-type and the unknown data points over the temperature, the relative differences were calculated. From these points the difference plot can be produced. If we used the wild type shape for reference, we can distinguish the alteration like SNP, short deletion, short deletion with SNP in heteroduplex, and longer deletions in different size (Figs. 4, 5, 6, 7, 8 and 9). In the case of SNPs mainly the same melting point with a different shape was created, resulting a peak at the end region of different plot. But in the case of deletions the start of the melting decreased, and the calculated difference value was higher in the first part of different plot. The longer the deletion is the earlier we get this higher ratio (Fig. 9).

Discussion

The melting curve shape analysis is very useful to detect any kind of sequence alterations inside the amplified region. This method are very easy to design and optimize. The critical value is the quality of sample DNA, degradation is important factor depending on the amplified size of the fragment. In this study the possible shortest fragment was used to avoid this problem. If the DNA can be amplified—it is analyzed with control reaction—the High Resolution Melting assay can distinguish the SNPs from the deletions and can be used to estimate the size of the deleted fragments. It is very fast and easy to use assay in order to check first our samples to select the none wild type sequences. Additionally we can see different and well distinguished shapes. The curve shapes can predict the type of alterations, equivalent or longer than one base.

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