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Multiplex Ligation-dependent Probe Amplification (MLPA) is not suitable for detection of low grade mosaicism.

Running title: Sensitivity of MLPA analysis.

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Abstract

“Apparent nonpenetrance” occurs in several genetic disorders, including Tuberous Sclerosis Complex and Neurofibromatosis type 1: clinically unaffected parents may have multiple affected offspring. Germ line or somatic mosaicism in one of the parents of the index patient is the probable cause and results in an enhanced recurrence risk. Therefore, it is of great importance to use the most sensitive technology for testing DNA of the parents of the index patient for the presence/absence of the familial mutation. To detect large rearrangements Multiplex Ligation-dependent Probe Amplification (MLPA) is often used. Here we show that MLPA is less sensitive in detecting low grade somatic mosaicism than Fluorescence *in situ* hybridisation (FISH) or a mutation-specific PCR test. Therefore we recommend FISH (if possible) or PCR analysis for the analysis of parental DNA.

Key Words

Sensitivity; MLPA; mosaicism; FISH; Mutation-specific PCR test; recurrence risk.

Introduction

Several genetic disorders, including Tuberous Sclerosis Complex and Neurofibromatosis type 1, show the effect of clinically unaffected parents who have multiple affected offspring. Germ line or somatic mosaicism in one of the parents has been described, resulting in a recurrence risk of up to 50%¹.

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder, caused by a mutation in either *TSC1* or *TSC2*, inactivating the protein². About 70 – 80% of the TSC patients are sporadic cases³. This observation does not exclude germ line mosaicism in one of the parents.

Neurofibromatosis type 1 (NF1) is an autosomal dominant neurocutaneous disorder⁴, caused by a mutation in *NF1*, resulting in an inactive protein.

Point mutations as well as large rearrangements occur in *TSC1*, *TSC2* and *NF1*. For the detection of large rearrangements, Multiplex Ligation-dependent Probe Amplification (MLPA) analysis is often used^{5,6}. No data about the sensitivity of MLPA analysis are available. Here we present data that MLPA is not suitable for the detection of low grade mosaicism. We recommend confirmation of an abnormal MLPA pattern by using either a mutation-specific PCR test after characterization of the breakpoint or, if possible, by Fluorescent in situ hybridization (FISH) analysis. These methods can be used for analysis of other family members, especially the parents, for a better risk assessment.

Materials and Methods

MLPA and FISH analysis

MLPA analysis was performed according to the manufacturer's instructions. Products were run on an automated sequencer (ABI 3730XL, Applied Biosystems, USA) and data were analysed using Genemarker version 1.5 (Softgenetics, USA). In our routine diagnostic setting, the analysis is based on the MLPA ratio. Quantification is based on the peak height and normalisation uses an Internal Control Probe. For sequences present in two copies in the sample, the resulting value is 1.0. A reduction or increase in peak height values results in values <0.70 or >1.30 , respectively (indicating a deletion or duplication). The kits used were: SALSA P046 for *TSC2* and SALSA P081/P082 for *NFI* (MRC-Holland BV, The Netherlands).

Because of the possibility of somatic mosaicism, at least 50 metaphases derived from cultured PHA-stimulated peripheral blood lymphocytes were analysed when performing FISH analysis for *NFI* using the probe cP5⁷.

Characterisation and confirmation of the breakpoint in TSC family.

Using long-range PCR analysis, the breakpoints of an observed MLPA abnormality were characterised by sequencing the aberrant PCR product. A gene defect-specific PCR test was developed using 3 primers, one common primer, one primer specific for the gene defect and one primer more 3' of the defect creating a wild type fragment that is larger than the mutant fragment. In this way, detection of the mutant signal is favoured (all primer sequences are available on request). The PCR products were run on an agarose gel.

Results and Discussion

Previously, an abnormal pattern using Southern blot analysis with cDNA probes of *TSC2* was obtained in the index patient of family 9703¹. DNA of the father showed a faint band at the same position of the abnormal fragment in DNA of the index patient, indicating that he was a somatic mosaic for his daughter's gene defect.

MLPA of *TSC2* showed a pattern consistent with a duplication of exon 15 – exon 26 (a probe for exon 27 is not present in the MLPA kit) (Figure 1C). Using our routine MLPA protocol, the duplication was hardly detectable in DNA of the father (Figure 1B). Using a range of paternal input DNA of 25 – 250 ng DNA (Figure 1J – 1L), no clearly visible duplication could be shown. Using the internal Control Probe Normalization as well as the Population Normalization using peak height as well as peak area calculation, also normalization and quantification of the peak results were tested. No differences between the results were seen (data not shown).

Long-range PCR, followed by sequencing of the abnormal product resulted in the identification of pathogenic mutation. The duplication started in intron 14 and extended to the middle of exon 27 of *TSC2*, combined with an insertion of CACG (c.[3232insCACG;1600-75_3232dup13997]).

For confirmation, a duplication specific PCR was designed and used for testing the family members. The abnormal fragment of 627 bp and the wild type fragment of 1315 bp were clearly visible in the index patient (Figure 2, lane 5). The duplication was not detected in control samples but was present in the father (Figure 2, lanes 1- 4). However, the ratio of

the mutant versus the wild type fragment was lower compared to the index patient, indicating that the father was somatic mosaic for the mutation, confirming the result of the Southern blot analysis.

To estimate the percentage of affected white blood cells of the father, a titration curve was prepared by mixing DNA of the index patient with a negative control (Figure 2, lanes 5 – 11). The duplication-specific PCR showed that about 30% of the father's leukocytes carried the duplication (Figure 2, lane 2).

The detection rate of the MLPA was compared to that of the duplication specific-PCR using the previously mixed samples. Figure 2 showed that the duplication-specific PCR was able to detect a mosaic pattern of about 10%. A mosaic pattern of about 40% could be detected by MLPA analysis (Figure 1C-11), which explains why the 30% of the father could not be detected by MLPA (Figure 1B).

To investigate the sensitivity of the MLPA in detecting deletions, 2 patients with a total *NFI* deletion previously identified using FISH analysis were tested. Patient 19578 carried the deletion in 85.4% of the cells and patient 26839 in 22%. MLPA analysis also showed the total gene deletions (Figures 3A and 3B). The sensitivity of the MLPA kit in detecting this *NFI* total gene deletion was determined by a titration analysis using DNA of family 19578. Using the NF1-1 MLPA kit P081, this deletion may be detected when it is present in about 30% of the cells (Figure 3A). The NF1-2 MLPA kit P082 showed the same sensitivity (data not shown). These analyses showed that it is possible to detect a deletion present in 22 – 30% of the cells. These results indicate that, using a quantitative analysis, mosaic duplications are harder to detect than deletions. The size of the rearrangement and the probe density can influence the detection. It can be difficult to identify mosaic

duplications or deletions of one exon, since the signal of the probe is also depending of DNA quality.⁸

It has been shown that MLPA analysis can be used to detect high grade mosaic *NF1* patients⁶. We investigated the sensitivity of the MLPA to detect a duplication of 7 exons of *TSC2* and total *NF1* deletions. Our results indicated that a low percentage of somatic mosaicism could be missed. Somatic mosaicism of a single exon deletion or duplication will be even harder to identify. We recommend to confirm abnormal MLPA patterns using an alternative technique; it is usually feasible to characterize the breakpoint and to develop a gene defect-specific PCR. Otherwise, for larger rearrangements, especially deletions, FISH analysis can be a reliable technique for the detection of low grade mosaicism. Depending on probe availability and the identified abnormality, BACs, cosmids or fosmids can be used as a probe. Another possibility, depending on the probe number in the area of interest, is to use an oligonucleotide or SNP-based array. It is known that High-Density SNP technology has a detection rate of up to ~30%⁹, which makes the sensitivity of this technique comparable to MLPA.

Detection of a low grade mosaic parent has great implications for genetic counseling. In case the pathogenic mutation can not be detected in DNA of the parents, a low recurrence risk on affected offspring will be given. If somatic mosaicism is identified in one of the parents, the recurrence risk may be up to 50%, indicating the necessity to use the most sensitive technologies for the analysis of parental DNA.

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Figure Legends

Figure 1: MLPA analysis of *TSC2*. The graphics showed the MLPA results of peak ratio vs size of: A, a negative control sample; B, father of the index patient; C, index patient; D – I, mixtures carrying the duplication in, respectively, 80% (D), 60% (E), 40% (F), 20% (G), 10% (H) and 0% of the cells (I); J-L, father of the index patient with a range of input DNA of, respectively, 100 ng (normal amount) (J), 25 ng (K) and 250 ng (L). (A blue dot refers to two copies of an internal control probe, a green dot to two copies of a gene specific probe and a red dot to respectively a deletion or duplication (<0.70 or >1.30) of a probe).

Figure 2: Agarose gel electrophoresis of the *TSC2* duplication specific PCR products. Lane 1, normal control; lane 2, father; lane 3 and lane 4, normal control; lane 5, index patient (100%); lane 6, 80% mosaic; lane 7, 60% mosaic; lane 8, 40% mosaic; lane 9, 20% mosaic; lane 10, 10% mosaic; lane 11, 0% mosaic; lane 12, no DNA.

Figure 3A: MLPA *NFI* results (only of the P081 kit). The graphics showed the MLPA results of peak ratio vs size. The percentage blood cells that carried the deletion varied from 85.4% to 0 % as indicated in the graphics.

Figure 3B: MLPA *NFI* results (only of the P081 kit) of a patient with a 22% mosaic total gene deletion.

(A bleu dot refers to two copies of an internal control probe, a green dot to two copies of a gene specific probe and a red dot to respectively a deletion or duplication (<0.70 or >1.30) of a probe).

Figure 1

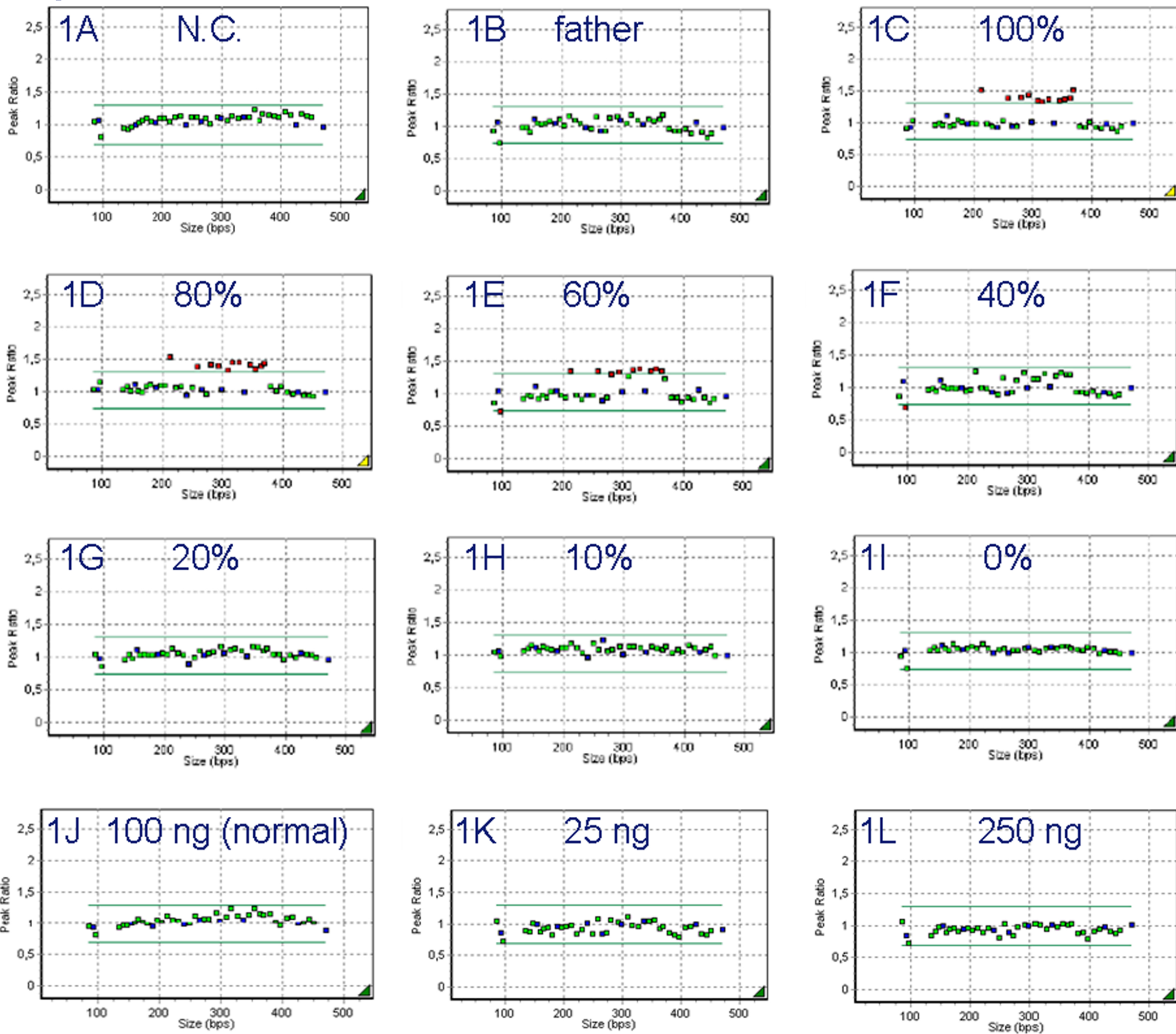


Figure 2

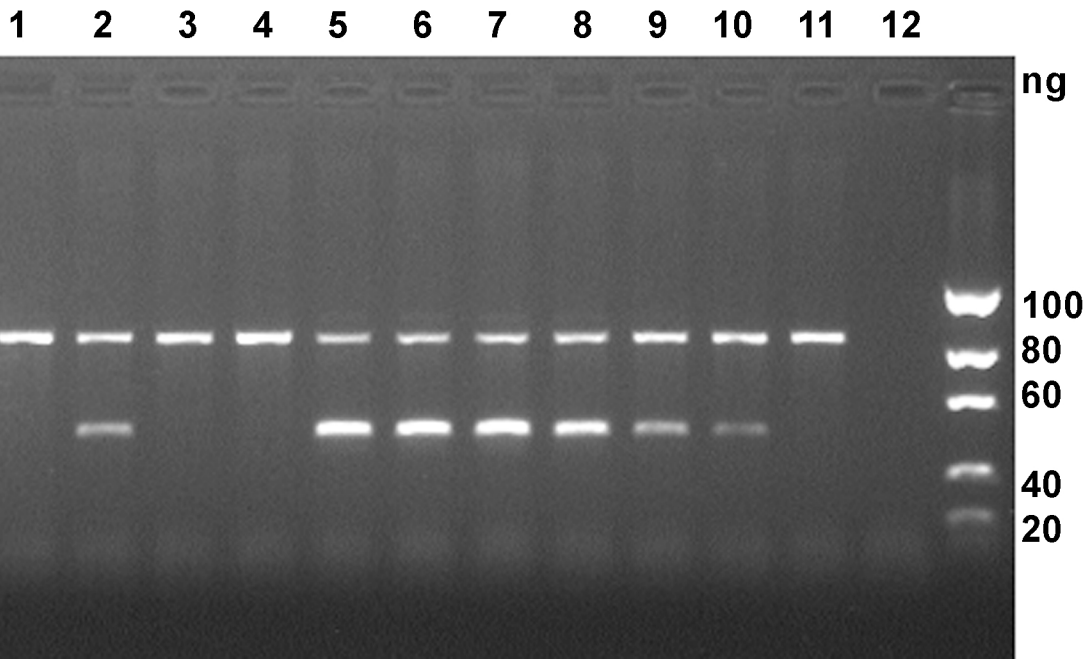


Figure 3A

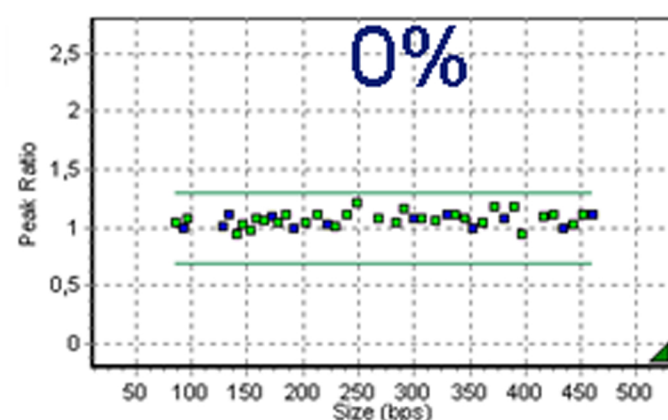
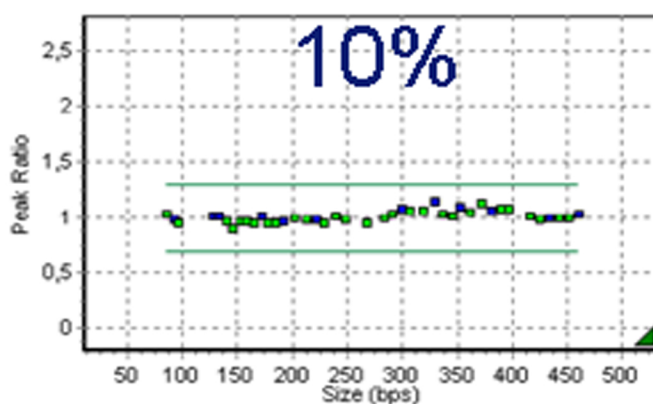
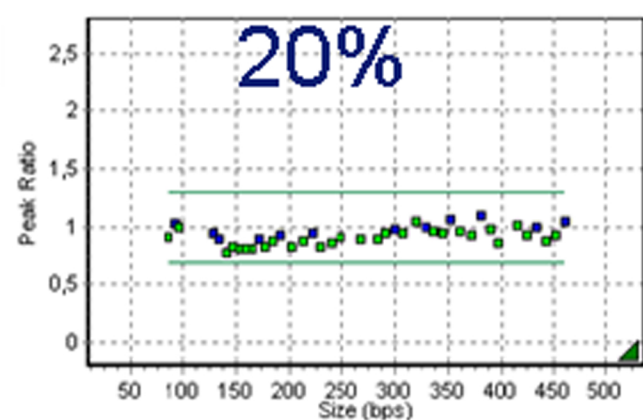
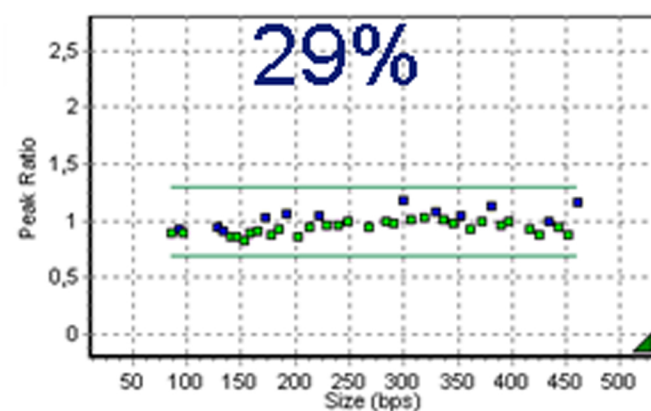
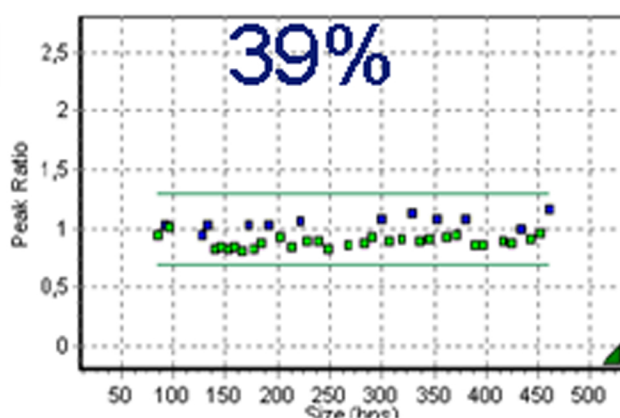
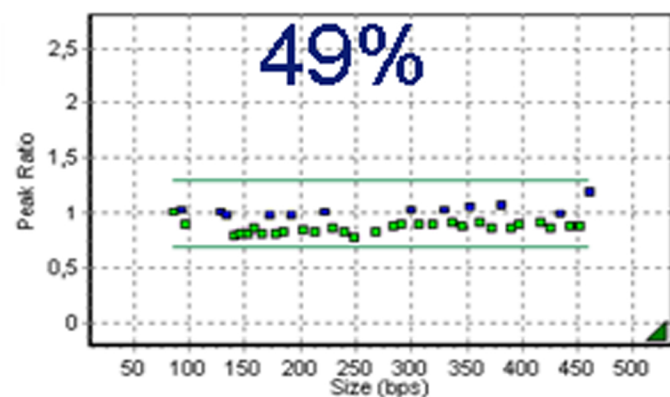
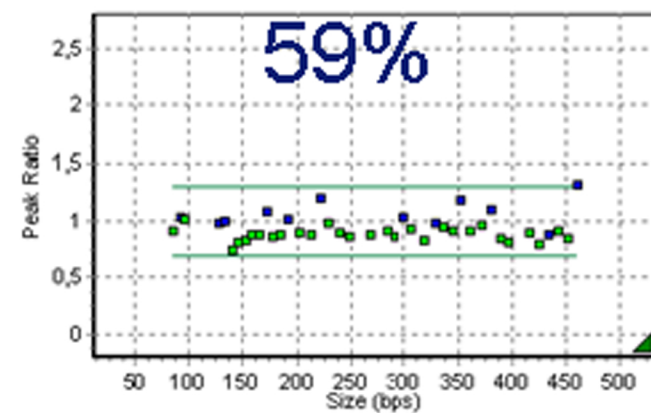
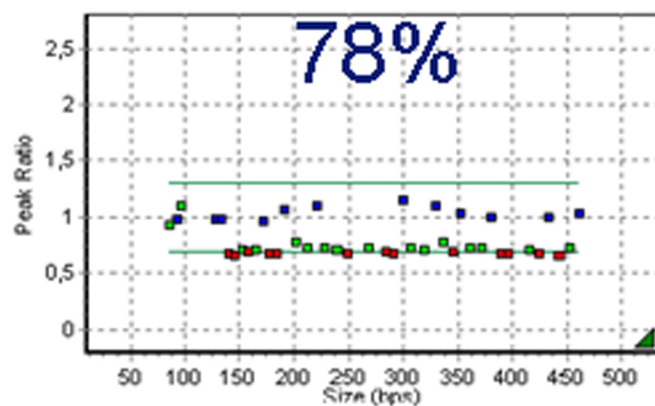
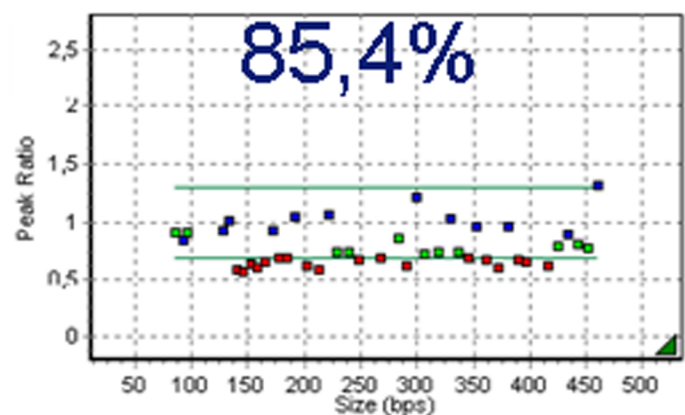


Figure 3B

