

## SALSA MLPA KIT P002B BRCA1

Lot 0107, 0706, 0606: As compared to previous lots, the exon 24 probe has been replaced.

Defects in the BRCA1 gene on human chromosome 17 are an important cause of hereditary breast cancer. Features characteristic of hereditary, versus sporadic, breast cancer are: younger age at diagnosis, frequent bilateral disease, and more frequent occurrence of disease among men. The probemix included in this kit contains probes for each exon of the BRCA1 gene. In addition, 9 probes for other human genes located on different chromosomes are included as a control.

In the Netherlands, more than 30 % of the BRCA1 related cases of hereditary breast cancer are due to copy number changes of one or more exons of this gene (Petrij-Bosch, A. et al. Nature Genet., 17: 341-345, 1997). The majority of these are due to two frequently occurring founder mutations: deletion of exon 13 or exon 22. Estimates in other countries are lower and range from 5 to 15 % (Unger, M.A. et al., Am.J.Hum.Genet. 67: 841-850, 2000). A wide variety of different exon deletions and duplications have been described. Exon deletions and amplifications will usually not be detected by sequence analysis of the complete BRCA1 gene. Known deletions and amplifications can be easily tested by PCR, but the number of different deletions is becoming prohibitively large. Analysis by MLPA is an easy to perform alternative that is also capable of detecting new deletions and amplifications.

In the future, each change in contents of this probemix will be reflected by a change in product number (P002B; P002C etc.).

**SALSA MLPA kit P087 BRCA1 can be used to confirm results obtained with this P002 probemix. We recommend using the P002 kit for the primary screening. P087 BRCA1 contains one probe for each BRCA1 exon. Distance between the ligation sites of the P087 probes and the corresponding P002 probes is at least 20 nucleotides, with the exception of exon 24 probe.**

The following MLPA kits may also be of interest:

P045 BRCA2/ CHEK2 - Breast cancer – genes included: BRCA2/ CHEK2

P190 CHEK2 – Breast cancer susceptibility – genes included: CHEK2, ATM, BRCA1&2, PTEN, XBP1, TP53

This MLPA kit is designed to detect deletions/duplications of one or more exons of the BRCA1 gene. Deletions of probe recognition sequences will be apparent by a 35-50% reduced relative peak area of the amplification product of that probe. However, mutations/polymorphisms very close to the probe ligation site may also result in a reduced relative peak area. Although we have tried to avoid BRCA1 mutation hot spots, the number of different mutations known is very large, and some mutations may affect relative peak areas. A 2 nt deletion in exon 14, which is next to the ligation site of the exon 14 probe did result in a 50 % reduction in relative probe signal for this probe. We suggest to sequence the particular exon in case only one exon appears to be deleted, or to confirm the deletion by PCR in case of e.g. the well-characterised Dutch exon 13 and 22 deletions. Deletion of the whole BRCA1 gene should be apparent by an increase in relative peak area of the 9 control bands. No deletions of the complete BRCA1 gene have been observed yet in MLPA screening of more than 1000 blood derived DNA samples from patients in which a hereditary cause of their breast cancer was suspected.

**MLPA kits are sold by MRC-Holland for research purposes and to demonstrate the possibilities of the MLPA technique. This kit is not CE/FDA certified for use in diagnostic procedures. SALSA MLPA kits are supplied with all necessary buffers and enzymes. Purchase of the SALSA MLPA test kits includes a limited license to use these products for research purposes.**

The use of this MLPA kit requires a thermocycler with heated lid and sequence type electrophoresis equipment. Different fluorescent PCR primers are available. The MLPA technique has been first described in Nucleic Acid Research 30, e57 (2002).

### More information

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### References of SALSA MLPA kit P002

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### Data analysis

The P002B probemix contains 34 different probes with amplification products between 127 and 454 nt, as well as 7 control fragments that generate amplification products smaller than 130 nt. Length difference between consecutive amplification products is 6 or 9 nt. Apparent deletions of a single exon should always be confirmed by other methods and / or by using the P087 MLPA probemix. The length of the amplification products does not include the 3' Adenine, which is usually added by a non-proofreading polymerase such as the SALSA polymerase.

MRC-Holland usually defines relative probe signals by dividing each measured peak area by the sum of all peak areas of that sample. The ratio of each peak's relative probe area is then compared to that obtained on a DNA control sample. Note that in case of large deletions, this method of analysis will result in a less than 50% reduction of relative probe signal. An alternative method is to define relative probe signals of probes by comparing them to the combined signals of the control probes. When only small numbers of samples are tested, visual comparison of peak profiles should be sufficient to easily identify exon deletions. Comparison of results should preferably be performed within one experiment, since intra-assay variation is larger. Only samples purified by the same method should be compared.

Apparent deletions of exons 1A + 1B +2 should be treated with caution, as contamination in the sample may prevent complete denaturation of the CpG islands near exons 1A, 1B and 2. The DD (DNA denaturation) control mix is added to this probemix to give a warning for incomplete denaturation. For more information, see control fragments.

Note that the Coffalyser MLPA-DAT, the MLPA analysis tool developed at MRC-Holland, can be downloaded free of charge from our website [www.mlpa.com](http://www.mlpa.com).

### Please note

BRCA1 exon 4 does not exist. Two alternative exons 1 exist: 1A and 1B. Probes for both exon 1A as well as 1B are included. Exon 1B is not present in the often used Genbank mRNA sequence U14680. Exon 11 is longer than all the other exons combined. We therefore made two probes for this exon 11, at a distance of 2800 nt. from each other.

A pseudogene containing a very similar copy of exons 1A, 1B and 2 is present on a short distance from the BRCA1 gene. The three MLPA probes for these exons should not generate a signal on this pseudogene as these probes have a mismatch at the probe ligation site when annealed to these pseudogene sequences. Relative probe signals for these three exons are higher than for most other probes. The probe signals of these three probes tend to be reduced when large amounts of DNA is used as compared to control reactions, and when PCR inhibitors are present in the DNA samples. If samples appear to have a deletion of these three exons, we recommend repeating the test with lower amounts of DNA, e.g. 40 ng.

In addition some contaminants can be present in DNA preparations which prevent complete denaturation of the CpG island close to exons 1A, 1B and 2. The DD control mix is added to this probe mix to give a warning for incomplete denaturation. For more information see control fragments.

### Control fragments

This P002 MLPA probemix contains four DQ (DNA Quantity) and three DD (DNA Denaturation) control fragments. The DQ control fragments can be recognized by the presence of 4 amplification peaks at regular distances, whose length always co-varies together (i.e. if one of the peaks is high, so are the other three). Amplification products of the MLPA DQ control fragments of 64, 70, 76 and 82 nt will be prominent if the amount of sample DNA is very low. These four fragments do not require a ligation reaction to be amplified, and are thus visible even when ligation has failed.

In contrast, the three MLPA DD control fragments of 88, 92 and 96 nt are DNA- and ligation dependent, as is the case with the normal MLPA probes. If the 64-70-76-82 nt DQ amplification products have similar or larger peak sizes than both the 88-92-96 nt fragments and the 130-472 nt ordinary MLPA probe amplification products, either the ligation reaction has failed or the amount of sample DNA was less than 20 ng. In either case, the results obtained may not be reliable.

The DD control fragments consist of two synthetic probe oligonucleotides and their purpose is to give a warning signal when the DNA has not been completely denatured. The peak size of the 92 nt amplification product in the DD control fragments reflects the copy number of a 2q14 DNA sequence. As said, the peak area of the 92 fragment should be of similar size as most of the other MLPA amplification products in the 130-454 nt range. Of the three DD control fragments, we deliberately added less of the 92 nt control probe, so that it becomes a measure for hybridization. If the peak size of the 92 nt amplification product is much lower than that of the 88 and 96 nt fragments of the DD control mix, this may indicate that the hybridization of probes to their targets has not been complete. Incomplete hybridization can be the result of the use of insufficiently long hybridization times; the use of insufficient amounts of probemix and/or MLPA buffer; the use of larger volumes of DNA solution (> 5 ul); a lower than 60 °C hybridization temperature; or a lower than 105 °C temperature of the thermocycler lid.

The 88 nt DD control fragment is specific for a sequence in the CpG island preceding the FANCE gene on 6p21.3. The 96 nt DD control probe is specific for a sequence within the CpG island preceding the TP73 gene on 1p36. These two CpG islands both include a long sequence with a very high percentage of C/G nucleotides, and are therefore very difficult to denature. In case the 88 and 96 nt amplification products are much lower than the 92 and 127-454 nt amplification products, denaturation of the sample DNA might have been incomplete, resulting in unreliable results for probes detecting sequences in or close (<5 Kb) to a CpG island. An extensive description of these control probes can be found in the MS-MLPA protocol.

This probemix was developed by Raymond Waaijer, Danny Zwijnenburg & J.P. Schouten at MRC-Holland. In case the results obtained with this probemix lead to a scientific publication, it would be very much appreciated if the probemix designer could be made a coauthor.

Info/remarks/suggestions for improvement: [info@mlpa.com](mailto:info@mlpa.com).

**SALSA MLPA P002B BRCA1 probemix**

Length (nt)	SALSA MLPA probe	Chromosomal position	
		Control	BRCA1
64-70-76-82*	DQ-control fragments		
88-92-96**	DD-control fragments		
127	Control probe 0797-L0093	5q31	
136	Control probe 0662-L0158	6p21	
<b>148</b>	<b>BRCA1 probe 0763-L0268</b>		<b>exon 1A</b>
<b>157</b>	<b>BRCA1 probe 0764-L0269</b>		<b>exon 1B</b>
<b>166</b>	<b>BRCA1 probe 0765-L0270</b>		<b>exon 2</b>
<b>175</b>	<b>BRCA1 probe 0826-L0341</b>		<b>exon 3</b>
<b>184</b>	<b>BRCA1 probe 0767-L0272</b>		<b>exon 5</b>
198	Control probe 2946-L3265	7q	
<b>208</b>	<b>BRCA1 probe 0827-L0342</b>		<b>exon 6</b>
<b>217</b>	<b>BRCA1 probe 0769-L0274</b>		<b>exon 7</b>
<b>226</b>	<b>BRCA1 probe 1004-L0569</b>		<b>exon 8</b>
<b>235</b>	<b>BRCA1 probe 1005-L0581</b>		<b>exon 9</b>
<b>244</b>	<b>BRCA1 probe 0772-L0277</b>		<b>exon 10</b>
256	Control probe 0518-L0098	2q14	
<b>268</b>	<b>BRCA1 probe 0830-L0345</b>		<b>exon 11</b>
<b>277</b>	<b>BRCA1 probe 0774-L0279</b>		<b>exon 11</b>
<b>286</b>	<b>BRCA1 probe 0775-L0280</b>		<b>exon 12</b>
<b>295</b>	<b>BRCA1 probe 2603-L2074</b>		<b>exon 13</b>
<b>304</b>	<b>BRCA1 probe 0833-L0349</b>		<b>exon 14</b>
316	Control probe 0495-L0303	12p12	
<b>328</b>	<b>BRCA1 probe 0778-L0347</b>		<b>exon 15</b>
<b>337</b>	<b>BRCA1 probe 0779-L0003</b>		<b>exon 16</b>
<b>346</b>	<b>BRCA1 probe 0780-L0283</b>		<b>exon 17</b>
<b>355</b>	<b>BRCA1 probe 0781-L0284</b>		<b>exon 18</b>
<b>364</b>	<b>BRCA1 probe 0782-L0285</b>		<b>exon 19</b>
376	Control probe 0655-L0304	4q26	
<b>388</b>	<b>BRCA1 probe 0783-L0356</b>		<b>exon 20</b>
<b>397</b>	<b>BRCA1 probe 0784-L0287</b>		<b>exon 21</b>
<b>406</b>	<b>BRCA1 probe 0785-L0288</b>		<b>exon 22</b>
<b>415</b>	<b>BRCA1 probe 0786-L0289</b>		<b>exon 23</b>
<b>424***</b>	<b>BRCA1 probe 2831-L2260</b>		<b>exon 24</b>
436	Control probe 0596-L0083	11p13	
445	Control probe 0678-L0124	12p13	
454	Control probe 0673-L0117	3p21	

\* Not ligation-dependent, these fragments indicate the amount of DNA used.

\*\* Ligation-dependent, these fragments give a warning for incomplete DNA denaturation.

\*\*\* **Present in this P002 probemix from lot 0606 onwards.**

**Please note: From lot 0606 (June 2006) onwards, the 424 nt exon 24 probe has been replaced. The probemix has been renamed P002B from lot 0606 onwards.**

The exon 24 probe that was used until May 2005 (0787-L0290), was reduced only 25% in signal on DNA samples with an exon 24 deletion (D. Bunyan, personal communication). The exon 24 probe that was used in lots 0805 and 1205 (Probe 4579-L3978) unfortunately resulted in the formation of an a specific fragment of 393 nt in some laboratories. This proved to be due to a 6 nucleotides homology between the probe sequence and the end of the MLPA forward PCR primer.

The exon 24 probe included from lot 0606 onwards (2831-L2260), is identical to the exon 24 probe in the P087 BRCA1 confirmation kit. In the future, each change in contents of this probemix will be reflected by a change in product number (P002B; P002C etc.).

## BRCA1 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	BRCA1 exon	Ligation site NM_007294.2	Sequence at Ligation site	Distance to next probe
		<i>startcodon</i>	<i>201-204</i>		
148	0763-L0268	exon 1A	160-159 Rev.	CAGAGGGTGA-AGGCCTCCTG	0.3 Kb
157	0764-L0269	exon 1B	3672-3673*	GGGGCACTGA-GTGTCGGTGG	1.0 Kb
166	0765-L0270	exon 2	216-217	TTTATCTGCTC-TTCGCGTTGA	8.3 Kb
175	0826-L0341	exon 3	303-304	AAGGAACCTG-TCTCCACAAA	9.3 Kb
184	0767-L0272	exon 5	361-362	TTCTCAACCA-GAAGAAAGGG	1.6 Kb
208	0827-L0342	exon 6	441-442	AGATTTAGTC-AACTTGTTGA	0.8 Kb
217	0769-L0274	exon 7	605-606	CCGTGCCAAA-AGACTTCTAC	4.4 Kb
226	1004-L0569	exon 8	686-687	TGGAACTGTG-AGAACTCTGA	2.6 Kb
235	1005-L0581	exon 9	781-782	TTAATAAGGC-AACTTATTGC	1.3 Kb
244	0772-L0277	exon 10	821-822	GTTACAAATC-ACCCCTCAAG	1.2 Kb
268	0830-L0345	exon 11	962-963	GCGTGCAGCT-GAGAGGCATC	2.8 Kb
277	0774-L0279	exon 11	3778-3779	CTAGCCCTTT-CACCCATACA	1.0 Kb
286	0775-L0280	exon 12	4345-4346	CTGAAGACTG-CTCAGGGCTA	8.5 Kb
295	2603-L2074	exon 13	4441-4442***	TGGCTGAACT-AGAAGCTGTG	5.9 Kb
304	0833-L0349	exon 14	4616-4617	AGAAGGCCTT-TCTGCTGACA	2.1 Kb
328	0778-L0347	exon 15	4750-4751	CTGGGAGTCT-TCAGAATAGA	3.2 Kb
337	0779-L0003	exon 16	4906-4907	CTGGAATCAG-CCTCTTCTCT	3.5 Kb
346	0780-L0283	exon 17	5214-5215	GCCAGAAAAC-ACCACATCAC	3.7 Kb
355	0781-L0284	exon 18	5294-5295	TGTGTGTGAA-CGGACACTGA	0.6 Kb
364	0782-L0285	exon 19	5369-5370	CCAGTCTATT-AAAGAAAGAA	6.3 Kb
388	0783-L0356	exon 20	5430-5431	GTCAATGGAA-GAAACCACCA	6.0 Kb
397	0784-L0287	exon 21	5504-5505	AATCTGTTGC-TATGGGCCCT	1.9 Kb
406	0785-L0288	exon 22	5578-5579	CTGTGGTGAA-GGAGCTTTCA	1.5 Kb
415	0786-L0289	exon 23	5622-5623	CACCCAATTG-TGGTTGTGCA	2.0 Kb
		<i>stopcodon</i>	<i>5790-5792</i>		
424	2831-L2260**	exon 24	6242-6243	AATGGAAGGA-GAGTGCTTGG	

\* Different Genbank #: L78833. Either exon 1A or 1B is used in transcripts.

\*\* **New from lot 0606 onwards.**

\*\*\* Sequence mentioned in version 3-8 of this P002 / P002B description was not correct. This exon 13 probe is present in the P002 probemix from August 2004 onwards.

Please note that the ligation sites mentioned are the locations in Genbank sequence NM\_007294.2, not the location from the startcodon.

We have been informed by Claire Morgan (Birmingham) that an apparent deletion of exon 19 proved to be due to the presence of the c.5276G>T (pVal1719Val) polymorphism.

Please notify us on any mistakes. The identity of the genes detected by the control probes is available on request: [info@mlpa.com](mailto:info@mlpa.com).

### Probes close to a CpG island

Apparent deletions detected by a single MLPA probe always require confirmation by other means. In general, the reduction in probe signal of two or more consecutive MLPA probes by more than 30% is a very strong indication that a true deletion is present. However, apparent deletions of two or more consecutive exons that are all located in close proximity of a strong CpG island have to be treated with caution. An example is the apparent deletion of BRCA1 exon 1A, 1B and 2. In case the sample DNA is incompletely denatured, all seven P002 and P087 probes for BRCA1 exons 1A, 1B and 2 may show reduced relative probe signals.

Complete denaturation of sample DNA is essential for all methods for quantification of specific DNA sequences. Denaturation of DNA is usually accomplished by heating the sample DNA. Before the start of MLPA reactions, the DNA is denatured by heating 5 minutes at 98 °C. Genomic DNA denatures at temperatures around 80 °C when the DNA is dissolved in TE (10 mM Tris-HC : 1 mM EDTA). The presence of higher concentrations of salt increases the denaturation temperature ( $T_m$ ) considerably. Besides, this  $T_m$  is dependent on the % C/G of the DNA.

The human genome contains thousands of sequences with a length of several hundreds to thousands of nucleotides, that have a very high % C/G. These CpG islands are often located around the transcription start sites of genes (close before exon 1) and are more difficult to denature than the rest of the genome.

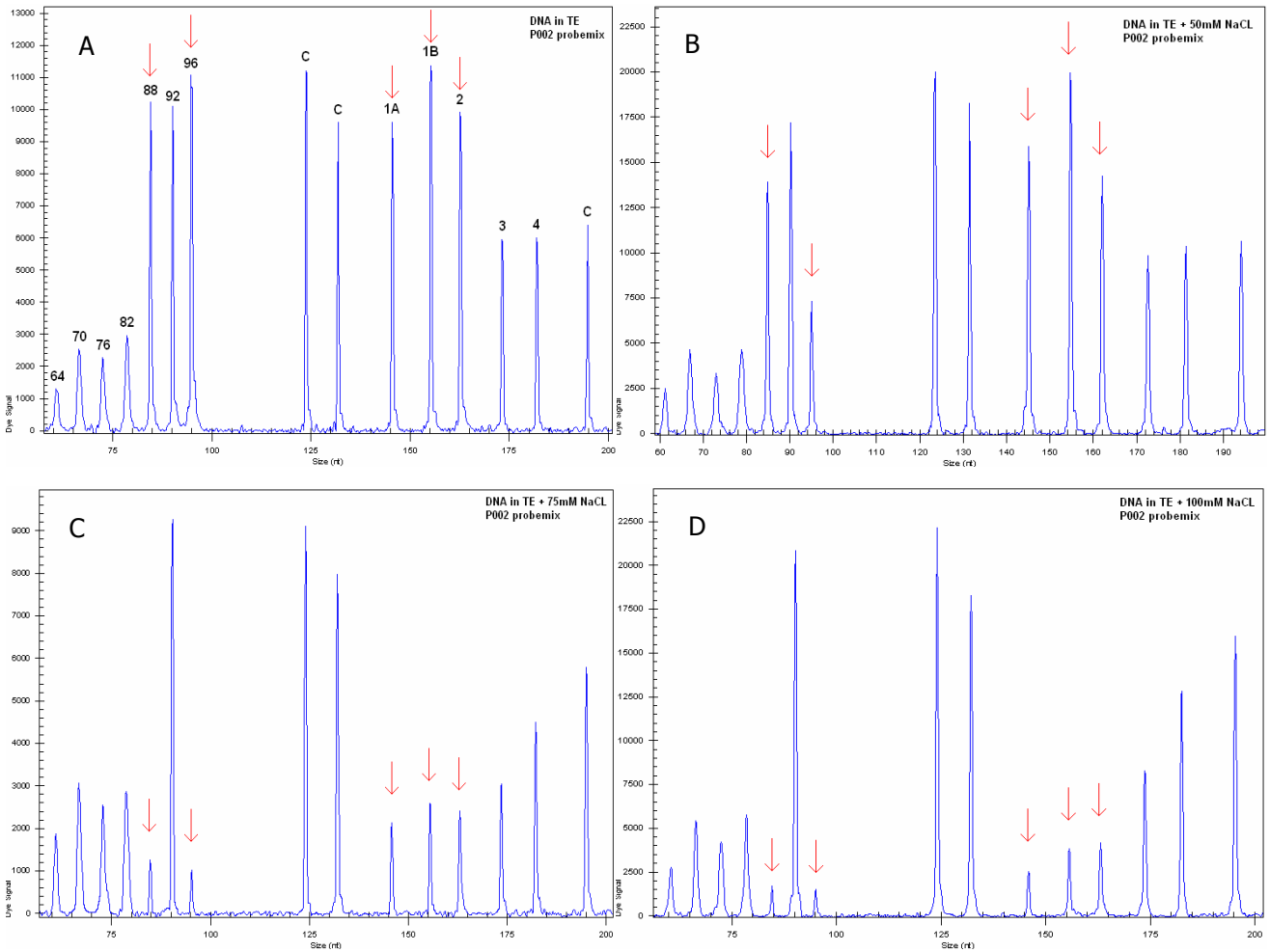
By heating the DNA for several minutes to 98 °C, as well as by pipetting, the size of the genomic DNA is usually reduced to fragments with an average size of 5.000-20.000 nucleotides. Incomplete denaturation of a part of such a long fragment (the CpG island) will keep the two complementary strands together and upon lowering of the temperature the two strands will immediately renature. As a result, a large part of the sequences that are located less than 5-10 Kb from a CpG island will not be available for binding of MLPA probes. These probes will generate a lower signal on incompletely denatured DNA as compared to probes recognising sequences located further away from CpG islands. As a result the apparent copy number of these incompletely denatured sequences seems to be lower compared to samples that are completely denatured. Renaturation of properly denatured, single copy, genomic DNA sequences requires many weeks of hybridisation.

In ordinary MLPA reactions, the size of the amplification products of the three control probes at 88, 92 and 96 nt will be similar (less than 50 % difference in peak height). However, the peak size of the 88 and 96 nt control fragments can be reduced, caused by incomplete denaturation due to CpG islands. As shown in Figure 1, the addition of up to 50 mM NaCl to a DNA sample in TE has no effect on the relative probe signals. At 75 mM NaCl however, the synthetic probes for the CpG islands of FANCE (88 nt) and TP73 (96 nt), as well as the probes for the first BRCA1 exons show a reduced relative probe signal. At 100 mM NaCl, the 88 and 96 nt control probes show a strongly reduced signal due to incomplete denaturation.

In general, most users will use only 1 µl of a DNA preparation and dilute this to 4 µl with TE for MLPA analysis. In order to obtain a salt concentration of 75 mM, the DNA should be 375 mM in salt when only 1 µl is used. Few DNA samples will have a salt concentration of > 375 mM. However, many other ions such as Mg have a much stronger effect on the  $T_m$  of DNA than NaCl at very low concentrations.

DNA preparations isolated by certain lots of commercial kits can give aberrant MLPA results. Impurities present in these DNA preparations may affect the PCR part of MLPA reactions. We expect that samples showing similar peak signals for the 88 and 96 nt control fragments as compared to the 92 nt and the 127-454 nt ordinary MLPA peaks are certainly completely denatured. In case the 88 and 96 nt control fragments apparently are not completely denatured, please try performing an MLPA reaction on lower amounts of sample DNA (lower amounts of ions / impurities) or try addition of 0.5 µl glycerol (resulting in a lower  $T_m$ ) to the MLPA reaction.

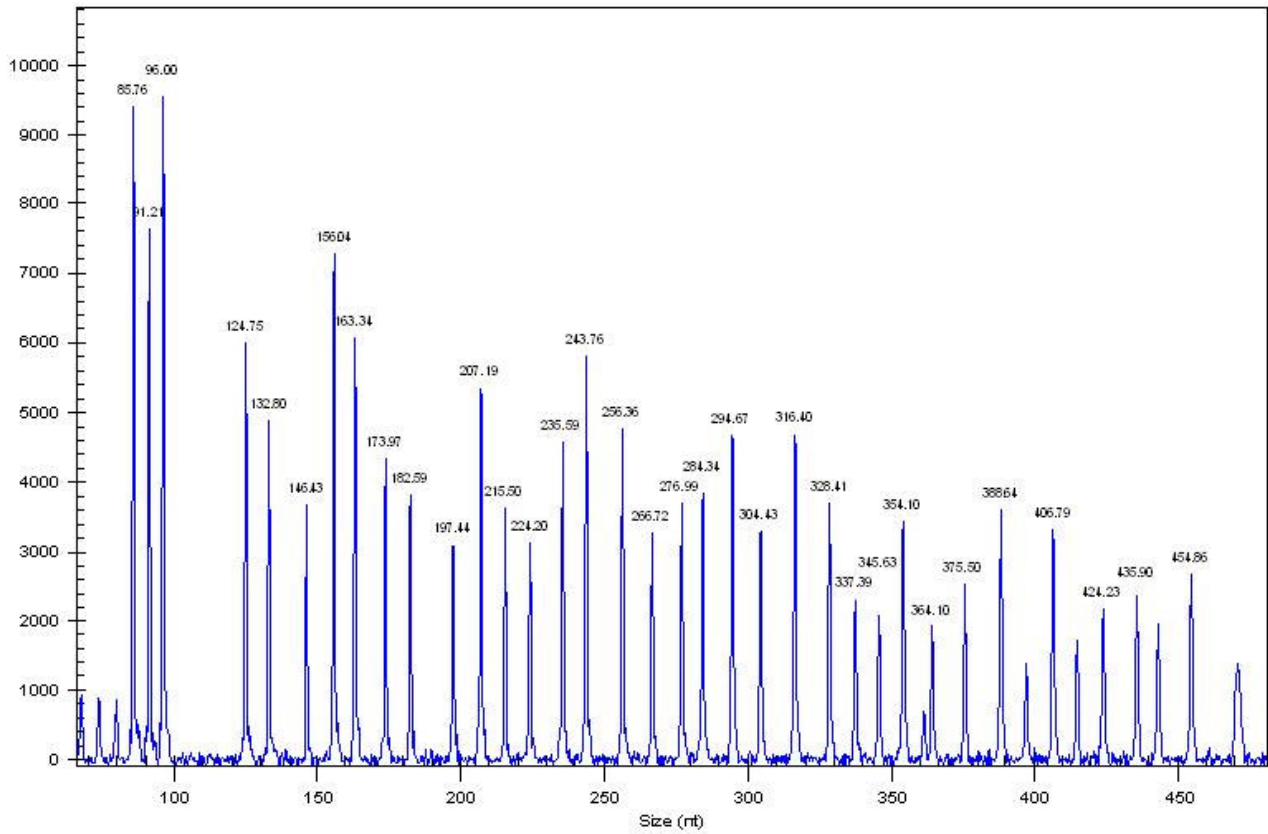
Please note that our results show that most CpG islands in human genomic DNA that is dissolved in ordinary 1 x PCR buffer (containing 50 mM KCl; 1.5 mM MgCl<sub>2</sub>) are not completely denatured by heating to 95 °C. Amplification products will be obtained by ordinary PCR even for sequences in CpG islands since a small part of the DNA is degraded and will be denatured. Real time PCR for the quantification of sequences located < 5-10 Kb from CpG islands will be unreliable if denaturation is performed in the presence of PCR buffer, as results will depend on the quality of the DNA. Degraded DNA will appear to have a higher copy number of sequences located close to CpG islands as compared to undegraded DNA. For PCR reactions of sequences close to CpG islands, we recommend to denature genomic DNA before the addition of PCR buffer. After denaturation, PCR buffer, dNTPs, primers and polymerase can be added.



**Figure 1:** MLPA P002 (old lot) probes signals from DNA in TE (A), with 50mM NaCl (B), 75 mM NaCl (C) and with 100 mM NaCl (D). Peak size of control fragments 88, 96 and probes for exon 1A, 1B and 2 are reduced in the presence of a high salt concentration due to incomplete denaturation. 64-96 nt: quality control fragments, c: control probe, 1-5: BRCA1 probe for exons 1-5.

## SALSA MLPA kit P002B BRCA1 sample picture

Lot 0107



**Figure 2.** Capillary electrophoresis pattern from a sample of approximately 50 ng human male DNA analyzed with SALSA MLPA kit P002 BRCA1 (lot 0107).