Product Description SALSA® MLPA® Probemix P235-B3 Retinitis

To be used with the MLPA General Protocol.

Version B3. As compared to version B2, one additional reference probe has been included, two reference probes have been replaced and one probe length has been adjusted. For complete product history see page 8.

Catalogue numbers:
- **P235-025R:** SALSA MLPA Probemix P235 Retinitis, 25 reactions.
- **P235-050R:** SALSA MLPA Probemix P235 Retinitis, 50 reactions.
- **P235-100R:** SALSA MLPA Probemix P235 Retinitis, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit and Coffalyser.Net data analysis software. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman/SCIEX capillary sequencers, respectively (see www.mlpa.com).

Certificate of Analysis: Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mlpa.com.

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions from findings generated with this product.

General information: The SALSA MLPA Probemix P235 Retinitis is a **research use only (RUO)** assay for the detection of deletions or duplications in the *RHO*, *IMPDH1*, *RP1*, and *PRPF31* genes, which are associated with Retinitis Pigmentosa (RP).

RP is a hereditary degenerative disease of the photoreceptor neurons of the retina. RP is characterized by progressive degeneration of the peripheral retina (leading to night blindness), loss of the peripheral visual field, and an abnormal electroretinogram. The genes most frequently involved in RP are *RHO*, *IMPDH1*, *RP1*, and *PRPF31*. Rhodopsin (RHO) is a highly-specialized G protein-coupled receptor that detects photons in the rod photoreceptors of vertebrates. Mutations in *IMPDH1*, a widely expressed rate-limiting enzyme of the de novo pathway of guanine nucleotide biosynthesis, have been shown to cause autosomal dominant RP. The gene for human oxygen regulated photoreceptor protein (*RP1*) encodes a protein that is localized in the connecting cilia of both rod and cone receptors. The RP1 protein is required for the morphogenesis of the outer segments of photoreceptor cells. Mutations in *RP1* cause at least 7% of autosomal dominant RP. Lastly, *PRPF31* encodes a 61 kDa protein which is essential for splicing in all cell types. The pathologic effect of mutations in this gene can be seen in rod photoreceptors. Furthermore, this probemix contains also a probe for *RPE65* gene which is also linked to RP.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1417/.

This SALSA MLPA Probemix is not CE/FDA registered for use in diagnostic procedures. Purchase of this product includes a limited license for research purposes.

Gene structure and transcript variants:
Entrez Gene shows transcript variants of each gene: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
For NM_ mRNA reference sequences: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Exon numbering: The *RHO*, *IMPDH1*, *RP1*, *PRPF31*, and *RPE65* exon numbering used in this P235-B3 Retinitis product description is the exon numbering from the RefSeq transcripts NM_000539.3, NM_000883.4, NM_006269.2, NM_015629.4, and NM_000329.3, which are identical to the NG_009115.1, NG_009194.1, NG_009840.2, NG_009759.1, and NG_008472.2 sequences. The exon numbering and NM_

sequences used have been retrieved on 05/2020. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Probemix content: The SALSA MLPA Probemix P235-B3 Retinitis contains 44 MLPA probes with amplification products between 130 and 463 nucleotides (nt). This includes five probes for the *RHO* gene, one probe for each exon, nine probes for the *IMPDH1* gene, targeting nine out of 17 exons, five exons for the *RP1* gene, one probe for each exon and an extra probe for exon 1, 15 probes for the *PRPF31* gene, one probe for each exon and two probes for exon 1, and one probe targeting exon 7 of the *RPE65* gene. In addition, nine reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mlpa.com).

This probemix contains nine quality control fragments generating amplification products between 64 and 105 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below). More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol and online at www.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal of 88 nt and 96 nt fragment indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

MLPA technique: The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mlpa.com).

MLPA technique validation: Internal validation of the MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. This validation experiment should result in a standard deviation ≤0.10 for all probes over the experiment.

Required specimens: Extracted DNA free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

Reference samples: A sufficient number (≥3) of reference samples should be included in each MLPA experiment for data normalisation. All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. Reference samples should be derived from unrelated individuals who are from families without a history of Retinitis Pigmentosa. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

Positive control DNA samples: MRC-Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (https://catalog.coriell.org) and Leibniz Institute DSMZ (https://www.dsmz.de/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample in your MLPA experiments. The quality of cell lines can change; therefore samples should be validated before use.

Data analysis: Coffalyser.Net software should be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net software is freely downloadable at www.mlpa.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

Interpretation of results: The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the dosage quotient (DQ) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the following cut-off values for the DQ of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Incomplete DNA denaturation (e.g. due to salt contamination) can lead to a decreased probe signal, in particular for probes located in or near a GC-rich region or in or near the *IMPDH1 gene*. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: lower injection voltage / injection time settings, or a reduced amount of sample by diluting PCR products.

Limitations of the procedure:
- In most populations, the major cause of genetic defects in the *RHO*, *IMPDH1*, *RP1*, and *PRPF31* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P235 Retinitis.
- MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.

Confirmation of results: Copy number changes detected by only a single probe always require confirmation by another method. An apparent deletion detected by a single probe can be due to e.g. a

mutation/polymorphism that prevents ligation or destabilises the binding of probe oligonucleotides to the DNA sample. Sequence analysis can establish whether mutations or polymorphisms are present in the probe target sequence. The finding of a heterozygous mutation or polymorphism indicates that two different alleles of the sequence are present in the sample DNA and that a false positive MLPA result was obtained.

Copy number changes detected by more than one consecutive probe should be confirmed by another independent technique such as long range PCR, qPCR, array CGH or Southern blotting, whenever possible. Deletions/duplications of more than 50 kb in length can often be confirmed by FISH.

LOVD mutation database: https://databases.lovd.nl/shared/genes. We strongly encourage users to deposit positive results in the Leiden Open Variation Database (LOVD). Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNPs and unusual results (e.g., a duplication of *RHO* exons 2 and 4 but not exon 3) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P235-B3 Retinitis

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]					
		Reference	RHO	RPE65	IMPDH1	RP1	PRPF31
64-105	Control fragments – see table in probemix content section for more information						
130	Reference probe 00797-L00463	5q31					
136	**PRPF31 probe** 06687-L07504						**Exon 1**
142	**PRPF31 probe** 06018-L05443						**Exon 8**
148	Reference probe 14279-L15949	15q13					
154	**PRPF31 probe** 06688-L05437						**Exon 2**
159	**RP1 probe** 03595-L02962					**Exon 1**	
166 «	**IMPDH1 probe** 10847-L11503				**Exon 17**		
172 «	**IMPDH1 probe** 10844-L11500				**Exon 2**		
177	**RHO probe** 03589-L07707		**Exon 1**				
184	**RP1 probe** 03594-L02961					**Exon 1**	
189 «	**IMPDH1 probe** 06989-L06591				**Exon 14**		
196	**PRPF31 probe** 10849-L11505						**Exon 9**
202	Reference probe 02213-L01217	20p12					
208	**PRPF31 probe** 11546-L12294						**Exon 3**
214	**RHO probe** 03590-L07200		**Exon 2**				
220	**RP1 probe** 03596-L11394					**Exon 2**	
229 «	**IMPDH1 probe** 11274-L11957				**Exon 1**		
238	Reference probe 17870-L22129	2p21					
246	**RHO probe** 03591-L02958		**Exon 3**				
256	**PRPF31 probe** 06020-L05445						**Exon 10**
263	**PRPF31 probe** 06014-L07202						**Exon 4**
270 «	**IMPDH1 probe** 11543-L13328				**Exon 12**		
276	**RPE65 probe** 03602-L02969			**Exon 7**			
283	**RHO probe** 11544-L12286		**Exon 4**				
292	**RP1 probe** 03597-L13368					**Exon 3**	
301	Reference probe 03255-L02692	11p13					
310	Reference probe 10863-L11533	9p21					
317	**RHO probe** 03593-L02960		**Exon 5**				
326	**RP1 probe** 03598-L13369					**Exon 4**	
337	**PRPF31 probe** 06021-L11393						**Exon 11**
346	**PRPF31 probe** 06015-L07508						**Exon 5**
353	**PRPF31 probe** 06022-L07203						**Exon 12**
364	**PRPF31 probe** 06016-L05441						**Exon 6**
371 «	**IMPDH1 probe** 21481-L30140				**Exon 6**		
381	**PRPF31 probe** 06023-L07204						**Exon 13**
391	**PRPF31 probe** 06017-L05442						**Exon 7**
400	**PRPF31 probe** 06024-L05449						**Exon 14**
414	**PRPF31 probe** 10848-L25118						**Exon 1**
421 «	**IMPDH1 probe** 06988-L25119				**Exon 9**		
427 «	**IMPDH1 probe** 06986-L06588				**Exon 3**		
436	Reference probe 10678-L11260	6p12					
445 «	**IMPDH1 probe** 11010-L11680				**Exon 5**		
454	Reference probe 12526-L23849	4q25					
463	Reference probe 11713-L12484	10q22					

a) See above section on exon numbering for more information.

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Table 2. P235-B3 probes arranged according to chromosomal location

Table 2a. *RHO*

Length (nt)	SALSA MLPA probe	RHO exon[a]	Ligation site NM_000539.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*96-98 (Exon 1)*		
177	03589-L07707	Exon 1	331-332	CCTGCTCAACCT-AGCCGTGGCTGA	2.0 kb
214	03590-L07200	Exon 2	554-555	GAGAACCATGCC-ATCATGGGCGTT	1.3 kb
246	03591-L02958	Exon 3	688-689	CAAGCCGGAGGT-CAACAACGAGTC	0.3 kb
283	11544-L12286	Exon 4	824-825	TCAGCCACCACA-CAGAAGGCAGAG	1.1 kb
317	03593-L02960	Exon 5	1059-1060	TCACCACCATCT-GCTGCGGCAAGA	
		stop codon	*1140-1142 (Exon 5)*		

Table 2b. *IMPDH1*

Length (nt)	SALSA MLPA probe	IMPDH1 exon[a]	Ligation site NM_000883.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*82-84 (Exon 1)*		
229 «	11274-L11957	Exon 1	124 nt before exon 2	CTAACTTCCCGAAACGCCACGCTC	0.1 kb
172 «	10844-L11500	Exon 2	249-250	GACCTCGCTACA-CACCCGACGACA	0.1 kb
427 «	06986-L06588	Exon 3	275-276	CCTCCTAGAACT-ATCTTCAGTGGT	5.6 kb
445 «	11010-L11680	Exon 5	476-477	CTTCATAGCTGA-TGAGGTGGTGAG	2.7 kb
371 «	21481-L30140	Exon 6	7 nt after exon 6, reverse	TGCAGGTGTGTA-ACTCACAGCCAT	0.8 kb
421 « #	06988-L25119	Exon 9	888-887, reverse	GCCACCACCAGT-TCAATCCTTGGC	3.7 kb
270 «	11543-L13328	Exon 12	94 nt after exon 12	GCCAGGCCCTGT-TCTGCCATGGTA	1.5 kb
189 « #	06989-L06591	Exon 14	1515-1514, reverse	GGGGCCTCCGTA-GTGGCGGCCAGC	1.9 kb
166 «	10847-L11503	Exon 17	121 nt before exon 17	CCGTACGGCTGA-GATCTCAGGGCT	
		stop codon	*1879-1881 (Exon 17)*		

Table 2c. *RP1*

Length (nt)	SALSA MLPA probe	RP1 exon[a]	Ligation site NM_006269.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*120-122 (Exon 2)*		
159	03595-L02962	Exon 1	77 nt before exon 1	TTCTGGCTGTTG-TCTCCTTAGGGT	0.1 kb
184	03594-L02961	Exon 1	15 nt before exon 1	CATACTGAGAAT-AAATCCAAAGAC	4.9 kb
220	03596-L11394	Exon 2	140-141	ACCCCTTCTACT-GGTTTTTCCATC	1.2 kb
292	03597-L13368	Exon 3	767-768	ATCCTGAGCTCT-GGAGCTGTGGTG	2.9 kb
326	03598-L13369	Exon 4	1319-1320	AGCAGTAATCAA-GAGGGCAGTTTG	
		stop codon	*6588-6590 (Exon 4)*		

Table 2d. *PRPF31*

Length (nt)	SALSA MLPA probe	PRPF31 exon[a]	Ligation site NM_015629.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*53-55 (Exon 2)*		
414	10848-L25118	Exon 1	276 nt before exon 1	GCTTAAAGGCCT-TGCTTTCTTGTC	0.2 kb
136	06687-L07504	Exon 1	25 nt before exon 1, reverse	TCTCGCGCCGTT-ATAGAGGCAAAG	2.7 kb
154	06688-L05437	Exon 2	198-199	GGATTCAGTCAA-GACCATCGCCAA	0.4 kb
208	11546-L12294	Exon 3	217 nt after exon 3	TGAGCTTACTGA-TCATGATAGGAC	3.1 kb
263	06014-L07202	Exon 4	364-365	GTGGAGATCGAA-AACGAGCTGAGT	0.6 kb
346	06015-L07508	Exon 5	454-455	AATGCACTGGAT-TACATCCGCACG	0.9 kb
364	06016-L05441	Exon 6	503-504	ACAAGTGCAAGA-ACAATGAGAACC	0.4 kb
391	06017-L05442	Exon 7	673-674	ATCTACGAGTAT-GTGGAGTCCCGG	0.8 kb
142	06018-L05443	Exon 8	852-853	GTCTACCTCAGT-GCTGCCCCACAC	1.9 kb
196	10849-L11505	Exon 9	43 nt before exon 9	TTACCTCTGTCT-GTCTGTCTCACA	1.6 kb
256	06020-L05445	Exon 10	1032-1033	GATCGAGCGCAA-ATTCGACAAGTG	0.3 kb
337	06021-L11393	Exon 11	1185-1186	GGCCAACCGTAT-GAGCTTCGGAGA	0.8 kb
353	06022-L07203	Exon 12	1288-1289	CAGACACAGGTA-AACGAGGCCACC	0.2 kb

Length (nt)	SALSA MLPA probe	PRPF31 exon[a]	Ligation site NM_015629.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
381	06023-L07204	Exon 13	1365-1366	ATATGGCGGGAA-GTCCACCATCCG	2.3 kb
400	06024-L05449	Exon 14	1632-1633	GGATCGGGTTCT-GGCAGGGAGAAC	
		stop codon	*1550-1552 (Exon 14)*		

Table 2e. *RPE65*

Length (nt)	SALSA MLPA probe	RPE65 exon[a]	Ligation site NM_000329.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*55-57 (Exon 1)*		
276	03602-L02969	Exon 7	726-727	GTCAGAGATCGT-TGTACAATTCCC	
		stop codon	*1654-1656 (Exon 14)*		

a) See above section on exon numbering for more information.

b) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Related SALSA MLPA probemixes

P221 LCA-1 Leber congenital amaurosis, genes included: *RPE65*, *AIPL1*, *CRB1*, and *CRX*.
P222 LCA-2 Leber congenital amaurosis, genes included: *CEP290*, *GUCY2D*, *RDH*, and *RPGRIP1*.

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
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Selected publications using SALSA MLPA Probemix P235 Retinitis

- Cathrine J et al. (2019). Molecular genetic analysis using targeted NGS analysis of 677 individuals with retinal dystrophy. *Sci Rep* (Nature Publisher Group), 9(1).
- de Sousa Dias M et al. (2015). New COL6A6 variant detected by whole-exome sequencing is linked to break points in intron 4 and 3'-UTR, deleting exon 5 of RHO, and causing adRP. *Mol vis*, 21, 857.
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- Eisenberger T et al. (2013). Increasing the yield in targeted next-generation sequencing by implicating CNV analysis, non-coding exons and the overall variant load: the example of retinal dystrophies. *PloS one, 8(11)*.
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- Golovleva I et al. (2010). Mutation spectra in autosomal dominant and recessive retinitis pigmentosa in northern Sweden. *Retinal Degenerative Diseases*. Springer New York. 255-262.
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P235 Product history	
Version	*Modification*
B3	One additional reference probe has been included, two reference probes have been replaced and one probe length has been adjusted.
B2	Two reference probes have been replaced and one added, in addition the control fragments have been adjusted (QDX2).
B1	Extra control fragments at 88, 96, 100, 105 nt. Three *IMPDH1* probes have been replaced (exons 2, 9, 17), three new *IMPDH* probes have been added (exons 5, 6, 12), and *IMPDH1* probe has been removed (exon 8). *RHO* exon 4 and *PRPF31* exon 1, 3, 9 have been replaced.
A1	First release.

Implemented changes in the product description

Version B3-01 — 31 July 2020 (02P)
- Product description rewritten and adapted to a new template.
- Ligation sites of the probes targeting the *RP1*, *PRPF31*, and *RPE65* genes updated according to new version of the NM_ reference sequence.
- Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.

Version 06 – 17 July 2017 (55)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included).
- New references added on page 2.
- Various minor textual changes on pages 1 and 2.

Version 05 (53)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included).

Version 04 (48)
- Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added.

More information: www.mlpa.com; www.mlpa.eu	
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