Product Description
SALSA® MLPA® Probemix P212-C3 DBA

To be used with the MLPA General Protocol.

Version C3
As compared to version C2, Three reference probes have been replaced and one probe length has been adjusted. For complete product history see page 8.

Catalogue numbers:
- **P212-025R:** SALSA MLPA Probemix P212 DBA, 25 reactions.
- **P212-050R:** SALSA MLPA Probemix P212 DBA, 50 reactions.
- **P212-100R:** SALSA MLPA Probemix P212 DBA, 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.mrcholland.com).

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

Precautions and warnings
For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mrcholland.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.

Note: In previous versions of the P212 DBA product description and in hg19, *RPS17* is annotated as a duplicated gene (*RPS17* and *RPS17L*), which was supported by several publications (e.g. Wat et al. 2010; Farrar et al. 2011). However, in the most recent hg38, and in a recent study (Ulisch et al. 2018), it is suggested that there is only a single location of *RPS17*.

General information
The SALSA MLPA Probemix P212 DBA is a **research use only (RUO)** assay for the detection of deletions or duplications in the *RPL11*, *RPL35A*, *RPS17*, *RPS19*, *RPS26*, and *RPL5* genes, which are associated with Diamond-Blackfan Anemia (DBA).

DBA is an erythroid aplasia characterised by normochromic macrocytic anemia, reticulocytopenia, and normocellular bone marrow with a selective deficiency of erythroid precursors. It usually becomes apparent during the first year of life and its symptoms include fatigue, weakness, and an abnormally pale appearance. Almost half of the patients show physical malformations involving head, thumb, heart, and urogenital system. Most cases are apparently sporadic, but in 10-25% of the cases the disease can be inherited with an autosomal dominant pattern. In the majority of these cases, DBA is caused by ribosomal protein haploinsufficiency due to mutations or deletions.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK7047/.

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 *RPL11*, *RPL35A*, *RPS17*, *RPS19*, *RPS26*, and *RPL5* exon numberings used in this P212-C3 DBA product description are the exon numberings from the LRG_1140, LRG_1129, LRG_1143, LRG_1144, LRG_1146, and LRG_1155 sequences, respectively. The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. As changes to the databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date.

Probemix content
The SALSA MLPA Probemix P212-C3 DBA contains 42 MLPA probes with amplification products between 130 and 447 nucleotides (nt). This includes one probe for each exon of the the *RPL11*, *RPL35A*, *RPS17*, *RPS19*, and *RPS26* genes and seven probes for the *RPL5* 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.mrcholland.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.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal 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.mrcholland.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 different unrelated individuals who are from families without a history of Diamond-Blackfan Anemia. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol (www.mrcholland.com).

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/) have diverse collections of biological resources which may be used as positive

control DNA samples 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.mrcholland.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 final ratio (FR) 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 FR of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	1.75 < FR < 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. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. 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.
- 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: a lower injection voltage or a shorter injection time, 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 *RPL11*, *RPL35A*, *RPS17*, *RPS19*, *RPS26*, and *RPL5* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P212 DBA.
- 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. SNVs, point mutations) in the target sequence detected by a probe can cause false positive results. Mutations/SNVs (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 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 SNVs and unusual results (e.g., a duplication of *RPL5* exons 4 and 6 but not exon 5) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P212-C3 DBA

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18) [a]						
		Reference	*RPL11*	*RPL35A*	*RPL5*	*RPS17*	*RPS19*	*RPS26*
64-105	Control fragments – see table in probemix content section for more information							
130	Reference probe 00797-L00463	5q						
136	**RPS19 probe** 17095-L20221						**Exon 3**	
142	**RPL5 probe** 18803-L24299				**Exon 7**			
148 *	Reference probe 19618-L26277	10p						
154	**RPS26 probe** 18804-L30009							**Exon 2**
160	**RPL5 probe** 18805-L24819				**Exon 5**			
166	**RPL11 probe** 18806-L24302		**Exon 4**					
173 Ж	**RPL35A probe** 18807-SP0700-L24303			**Exon 2**				
179 ∫	**RPS17 probe** 18808-L24304					**Exon 3**		
185	**RPL5 probe** 18809-L30010				**Exon 2**			
192	**RPS19 probe** 17099-L20225						**Exon 6**	
197 ¥	Reference probe 15412-L30011	7p						
202 Ж	**RPL11 probe** 18810-SP0701-L30012		**Exon 5**					
210	**RPL5 probe** 18811-L24307				**Exon 8**			
214	**RPL35A probe** 18812-L30013			**Exon 4**				
220	**RPS19 probe** 21348-L30014						**Exon 2**	
229 ∫	**RPS17 probe** 18813-L24309					**Exon 2**		
238	**RPL5 probe** 18814-L24310				**Exon 4**			
244	**RPS26 probe** 18815-L24311							**Exon 3**
251 *	Reference probe 21321-L29727	3p						
258	**RPS19 probe** 17101-L30015						**Exon 5**	
265 Ж	**RPL35A probe** 18816-SP0702-L24312			**Exon 5**				
274	**RPL11 probe** 18817-L24313		**Exon 3**					
283	**RPS19 probe** 17102-L20228						**Exon 4**	
292	**RPS26 probe** 18818-L24314							**Exon 1**
301 ∫	**RPS17 probe** 18819-L24315					**Exon 5**		
309	**RPS19 probe** 17082-L06753						**Exon 1**	
319	Reference probe 10084-L10508	8q						
328	**RPL35A probe** 18820-L24316			**Exon 3**				
337	**RPL5 probe** 18821-L24317				**Exon 1**			
346 Ж	**RPL11 probe** 18822-SP0703-L24318		**Exon 6**					
355 ∫	**RPS17 probe** 18823-L24319					**Exon 1**		
364	Reference probe 08740-L08751	9q						
371	**RPL35A probe** 18824-L24320			**Exon 1**				
383	**RPS26 probe** 18825-L24321							**Exon 4**
393 Ж	**RPL11 probe** 18826-SP0704-L24322		**Exon 2**					
402	**RPL5 probe** 18827-L24323				**Exon 6**			
409	Reference probe 02669-L02136	11q						
418	**RPL11 probe** 18828-L24324		**Exon 1**					
427 ∫	**RPS17 probe** 18829-L24325					**Exon 4**		
436	Reference probe 19990-L30109	4p						
447 *	Reference probe 20058-L30698	16q						

[a] See section Exon numbering on page 2 for more information.

* New in version C3.

¥ Changed in version C3. Minor alteration, no change in sequence detected.

Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

∫ Important information on this probe can be found in and below Table 2.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Table 2. P212-C3 probes arranged according to chromosomal location

Table 2a. *RPL11*

Length (nt)	SALSA MLPA probe	*RPL11* exon[a]	Ligation site NM_000975.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*19-21 (Exon 1)*		
418	18828-L24324	Exon 1	23-24	CTCCATCATGGC-GGTGAGTAGCTG	0.8 kb
393 Ж	18826-SP0704-L24322	Exon 2	75-76 and 103-104	CGCATCCGCAAA-28 nt spanning oligo-GTGGAGACAGAC	1.2 kb
274	18817-L24313	Exon 3	279-280	GAGAAGGGTCTA-AAGGTGAGCCTA	0.8 kb
166	18806-L24302	Exon 4	287-288	TCCACAGGTGCG-GGAGTATGAGTT	1.2 kb
202 Ж	18810-SP0701-L30012	Exon 5	491-492 and 521-522	ACACAGAATCAG-30 nt spanning oligo-GAAGGTAAAGCT	0.5 kb
346 Ж	18822-SP0703-L24318	Exon 6	577-578 and 616-617	CTATCCAAAAGA-39 nt spanning oligo-GTGTTCTGTGAA	
		stop codon	*553-555 (Exon 6)*		

Table 2b. *RPL5*

Length (nt)	SALSA MLPA probe	*RPL5* exon[a]	Ligation site NM_000969.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*76-78 (Exon 1)*		
337	18821-L24317	Exon 1	74-73, reverse	CACTCACCATCC-TGCGGAACAGAG	1.4 kb
185	18809-L30010	Exon 2	48 nt after exon 2	GCTTTGCAGATG-CAGTGGAGTATC	1.3 kb
	No probe	*Exon 3*			
238	18814-L24310	Exon 4	270-271	CTATAGATTGCT-TATGCCCGTATA	1.6 kb
160	18805-L24819	Exon 5	602-603	TATCCCTCACAG-GTAAGAATACTA	1.2 kb
402	18827-L24323	Exon 6	776-777	CGTAACTCCAGA-CATGGTAAAACA	2.9 kb
142	18803-L24299	Exon 7	784-785	TGTTTCAGATGG-AGGAGATGTATA	1.5 kb
210	18811-L24307	Exon 8	163 nt after exon 8	TTACCTGACAGG-AGTCATTATCCC	
		stop codon	*967-969 (Exon 8)*		

Table 2c. *RPL35A*

Length (nt)	SALSA MLPA probe	*RPL35A* exon[a]	Ligation site NM_000996.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*65-67 (Exon 2)*		
371	18824-L24320	Exon 1	20 nt before exon 1	AATCCCACAAGG-CCACGCGGCGGG	0.7 kb
173 Ж	18807-SP0700-L24303	Exon 2	50 nt and 14 nt before exon 2	AGGGGACGAGGT-36 nt spanning oligo-TTGTTTGTTTTA	0.4 kb
328	18820-L24316	Exon 3	222-223	TGTATATAAAGC-AAAGAAGTAAGT	2.9 kb
214	18812-L30013	Exon 4	33 nt after exon 4	ATGGACGTCTGA-TGAATCAGACTA	1.7 kb
265 Ж	18816-SP0702-L24312	Exon 5	469-470 and 514-515	AAAACTTACTAC-45 nt spanning oligo-CATTTTTGGAGT	
		stop codon	*395-397 (Exon 5)*		

Table 2d. *RPS26*

Length (nt)	SALSA MLPA probe	*RPS26* exon[a]	Ligation site NM_001029.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*250-252 (Exon 1)*		
292	18818-L24314	Exon 1	16 nt before exon 1	CAAGGCTTGCTG-GGAGACACATAA	0.5 kb
154	18804-L30009	Exon 2	3 nt before exon 2	CCCATCCTGTCG-CAGACAAAGAAA	0.9 kb
244	18815-L24311	Exon 3	438-439	TTAGCCTATGTG-CTTCCCAAGCTG	0.8 kb
383	18825-L24321	Exon 4	566-567	CTTTCAGGGTGC-TGCCCCACGTCC	
		stop codon	*595-597 (Exon 4)*		

Table 2e. *RPS17*

Length (nt)	SALSA MLPA probe	*RPS17* exon[a]	Ligation site NM_001021.6	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*30-32 (Exon 1)*		
355 ∫	18823-L24319	Exon 1	31-32	ACCCGCCAACAT-GGTAGGTGTTTT	0.3 kb
229 ∫	18813-L24309	Exon 2	4 nt before exon 2	TCCTCTCCACCC-GCAGGGCCGCGT	1.3 kb
179 ∫	18808-L24304	Exon 3	17 nt after exon 3	CTTTCTGGATAT-TTGGGCTTCTGG	0.6 kb
427 ∫	18829-L24325	Exon 4	3 nt after exon 4	AAGCTTTTGGTA-AGTGTTTGCTGG	1.6 kb
301 ∫	18819-L24315	Exon 5	53 nt after exon 5	CAGGAGCTCTCG-AGGCAACAGATC	
		stop codon	*435-437 (Exon 5)*		

Table 2f. *RPS19*

Length (nt)	SALSA MLPA probe	*RPS19* exon[a]	Ligation site NM_001022.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*36-38 (Exon 2)*		
309	17082-L06753	Exon 1	299 nt before exon 1	ACCACTGTTCCT-TCCAGCCACGAA	0.8 kb
220	21348-L30014	Exon 2	1 nt before exon 2	CTTTCTCCCTCA-GATGCCTGGAGT	0.6 kb
136	17095-L20221	Exon 3	118 nt after exon 3	AAACCCTTGGTT-GTGTCACCACTT	7.7 kb
283	17102-L20228	Exon 4	43 nt before exon 4	GTTTACCTGAGA-CCTTGATCAAGA	0.6 kb
258	17101-L30015	Exon 5	140 nt before exon 5	CAGCTCGTTAGA-ATGCACCTGACT	1.7 kb
192	17099-L20225	Exon 6	72 nt before exon 6, reverse	AACAGCTACCTA-ACTACCCACCAC	
		stop codon	*471-473 (Exon 6)*		

[a] See section Exon numbering on page 2 for more information.
[b] Only partial probe sequences are shown. Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

∫ In previous versions of the P212 DBA product description and in hg19, *RPS17* is annotated as a duplicated gene (*RPS17* and *RPS17L*), which was supported by several publications (e.g. Wat et al. 2010; Farrar et al. 2011). However, in the most recent hg38, and in a recent study (Ulisch et al. 2018), it is suggested that there is only a single location of *RPS17*.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

References

- Farrar JE et al. (2012). Ribosomal protein gene deletions in Diamond-Blackfan anemia. *Blood.* 118:6943-51.
- 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.
- Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem.* 421:799-801.
- Ulirsch JC et al. (2018). The Genetic Landscape of Diamond-Blackfan Anemia. *Am J Hum Genet*. 103:930-47.
- Wat MJ et al. (2010). Recurrent microdeletions of 15q25.2 are associated with increased risk of congenital diaphragmatic hernia, cognitive deficits and possibly Diamond-Blackfan anaemia. *J Med Genet*. 47:777-81.

Selected publications using SALSA MLPA Probemix P212 DBA

- Quarello P et al. (2008). Multiplex ligation-dependent probe amplification enhances molecular diagnosis of Diamond-Blackfan anemia due to RPS19 deficiency. *Haematologica*. 93:1748-50.

P212 product history	
Version	*Modification*
C3	Three reference probes have been replaced and one probe length has been adjusted.
C2	Two reference probes have been replaced and several probe lengths have been adjusted.
C1	The probes for the chromosome 8 genes *CSMD1*, *ANGPT2* and *AGPAT5* have been removed and the mix now contains probes for the ribosomal protein encoding genes *RPL11*, *RPL5*, *RPL35A*, *RPS26*, *RPS17* and *RPS19*.
B1	Ten probes have been replaced and six new reference probes have been added/replaced.
A2	The 88, 96, 100 and 105 nt control fragments have been included.
A1	First release.

Implemented changes in the product description

Version C3-01 – 23 March 2021 (04P)
- Product description rewritten and adapted to a new template.
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).
- Ligation sites of the probes targeting the *RPS19* gene updated according to new version of the NM_ reference sequence.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.

Version C2-01 – 24 January 2019 (01P)
- Product description restructured and adapted to a new template.
- Ligation sites of the majority of the probes updated according to new version of the NM_reference sequence.
- Information regarding the normal copy number of the *RPS17* gene included.

Version 14 – 07 September 2017 (55)
- Warning added in Table 1, 136 nt probe 17095-L20221 and 220 nt probe 21348-L30014.

Version 13 – 06 June 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).

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