Product Description
SALSA® MLPA® Probemix P409-B1 RASA1-EPHB4

To be used with the MLPA General Protocol.

Version B1
For complete product history see page 8.

Catalogue numbers:
- **P409-025R:** SALSA MLPA Probemix P409 RASA1-EPHB4, 25 reactions.
- **P409-050R:** SALSA MLPA Probemix P409 RASA1-EPHB4, 50 reactions.
- **P409-100R:** SALSA MLPA Probemix P409 RASA1-EPHB4, 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.

General information
The SALSA MLPA Probemix P409 RASA1-EPHB4 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *RASA1* and *EPHB4* genes, which are associated with capillary malformation-arteriovenous malformation (CM-AVM) syndrome.

CM-AVM syndrome is a disorder of the vascular system that is characterised by multiple cutaneous capillary malformations (CMs) that are mostly found on the face, arms and legs. Some affected individuals also have one or more arteriovenous malformations (AVMs) and/or arteriovenous fistulas (AVFs), fast-flow vascular malformations that typically arise in the skin, muscle, bone, spine and brain. Depending on their location in the body, these abnormalities could lead to life-threatening complications including haemorrhage and heart failure. The presence of AVMs/AVFs can also cause soft tissue and skeletal hypertrophy, resulting in limb overgrowth. This phenotype of CM-AVM syndrome is called Parkes Weber syndrome.

CM-AVM is an autosomal-dominant disease that is caused by mutations in the *RASA1* and *EPHB4* genes (Eerola et al. 2003; Amyere et al. 2017). The *RASA1* gene encodes Ras GTPase-activating protein 1, a protein involved in a wide variety of processes including regulation of cellular differentiation and proliferation, and cytoskeletal reorganisation. Ras GTPase-activating protein 1 is thought to have an important function in the development of the vascular system. The *RASA1* gene (25 exons) spans ~124 kb of genomic DNA and is located on chromosome 5q14.3, about 87 Mb from the p-telomere. The *EPHB4* gene encodes the ephrin type-B receptor 4, a transmembrane receptor that is expressed in venous endothelial cells during vascular development. Mutations in *EPHB4* hamper EPHB4-EphrinB2 signalling, which leads to abnormal differentiation of endothelial cells and disorganized vascular development. The *EPHB4* gene (17 exons) spans ~25 kb of genomic DNA and is located on chromosome 7q22.1, about 100 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK52764/.

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 *RASA1* and *EPHB4* exon numbering used in this P409-B1 RASA1-EPHB4 product description is the exon numbering from the NG_011650.1 and NG_052671.1 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 NG 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 P409-B1 RASA1-EPHB4 contains 50 MLPA probes with amplification products between 124 and 499 nucleotides (nt). This includes 26 probes for the *RASA1* gene, one probe for each exon and two probes for exon 1, and 16 probes for the *EPHB4* gene, one probe for each exon with the exception of exon 10. In addition, eight 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-fragments (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 capillary malformations. 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

Note: The term “dosage quotient”, used in older product description versions, has been replaced by “final ratio” to become consistent with the terminology of the Coffalyser.Net software. (Calculations, cut-offs and interpretation remain unchanged.) Please note that the Coffalyser.Net software also shows arbitrary borders as part of the statistical analysis of results obtained in an experiment. As such, arbitrary borders are different from the final ratio cut-off values shown here above.

- 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 or in or near the *EPHB4* 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.
- 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 *RASA1* and *EPHB4* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P409 RASA1-EPHB4.
- 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.

***RASA1* and *EPHB4* mutation databases**

http://arup.utah.edu/database/RASA1/RASA1_display.php, https://databases.lovd.nl/shared/genes/RASA1 and https://databases.lovd.nl/shared/genes/EPHB4. We strongly encourage users to deposit positive results in the ARUP RASA1 Database and 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 *RASA1* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P409-B1 RASA1-EPHB4

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*RASA1*	*EPHB4*
64-105	Control fragments – see table in probemix content section for more information			
124	Reference probe 19616-L26241	4p		
130	**EPHB4 probe** 22094-L31081			**Exon 16**
136 «	**EPHB4 probe** 22095-L31420			**Exon 2**
142	**RASA1 probe** 17231-L20567		**Exon 17**	
149	**RASA1 probe** 17232-L20568		**Exon 1**	
154	**RASA1 probe** 17233-L20569		**Exon 25**	
160	**RASA1 probe** 22092-L21513		**Exon 7**	
166	**RASA1 probe** 17234-L20570		**Exon 6**	
173	Reference probe 19185-L27754	3q		
178	**RASA1 probe** 17236-L20572		**Exon 23**	
184 «	**EPHB4 probe** 22096-L31083			**Exon 1**
190	**RASA1 probe** 17237-L20573		**Exon 2**	
196 Ж	**RASA1 probe** 17238-SP0462-L20574		**Exon 13**	
202	**RASA1 probe** 17239-L20575		**Exon 4**	
208	Reference probe 16261-L18553	20q		
215	**RASA1 probe** 17416-L21135		**Exon 16**	
220	**EPHB4 probe** 22097-L31084			**Exon 15**
226	**EPHB4 probe** 22098-L31085			**Exon 17**
232 «	**EPHB4 probe** 22099-L31086			**Exon 6**
240	**RASA1 probe** 17243-L31376		**Exon 12**	
247	Reference probe 21928-L30731	15q		
256	**RASA1 probe** 17245-L20581		**Exon 24**	
261 «	**EPHB4 probe** 22101-L31378			**Exon 7**
268	**EPHB4 probe** 22102-L31089			**Exon 14**
275	**RASA1 probe** 17246-L21515		**Exon 9**	
285	**RASA1 probe** 17247-L31377		**Exon 14**	
292 «	**EPHB4 probe** 22103-L31090			**Exon 5**
301	Reference probe 14941-L16674	6q		
310 Ж	**RASA1 probe** 17249-SP0464-L21516		**Exon 1**	
319	**RASA1 probe** 17250-L20586		**Exon 22**	
328	**RASA1 probe** 17251-L20587		**Exon 10**	
334 «	**EPHB4 probe** 22104-L31091			**Exon 3**
346	**RASA1 probe** 17252-L20588		**Exon 19**	
356	**RASA1 probe** 17253-L20589		**Exon 8**	
364	**RASA1 probe** 17254-L20590		**Exon 15**	
372	Reference probe 14422-L16127	12q		
384	**RASA1 probe** 17255-L20591		**Exon 18**	
391	**RASA1 probe** 17256-L20592		**Exon 3**	
402	**RASA1 probe** 17257-L20593		**Exon 21**	
409 «	**EPHB4 probe** 22105-L31092			**Exon 8**
418 «	**EPHB4 probe** 22106-L31093			**Exon 4**
427	**EPHB4 probe** 22107-L31094			**Exon 11**
436	**RASA1 probe** 22037-L30961		**Exon 5**	
445	Reference probe 12002-L23932	8q		
454	**EPHB4 probe** 22108-L31095			**Exon 13**
463	**EPHB4 probe** 22109-L31096			**Exon 9**
472	**RASA1 probe** 22066-L20571		**Exon 11**	
481 Ж	**RASA1 probe** 22091-SP0463-L21514		**Exon 20**	
493	**EPHB4 probe** 22110-L31097			**Exon 12**
499	Reference probe 09870-L15194	2p		

[a] See section Exon numbering on page 2 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.

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.

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. P409-B1 probes arranged according to chromosomal location

Table 2a. *RASA1*

Length (nt)	SALSA MLPA probe	*RASA1* exon[a]	Ligation site NM_002890.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*570-572 (Exon 1)*		
310 Ж	17249-SP0464-L21516	Exon 1	749-750; 776-777	GTGGCTGGAACT-27 nt spanning oligo-GAGTTCCTAGGA	0.2 kb
149	17232-L20568	Exon 1	910-911	TAGTGGAGACAT-GGCTCTCACCAA	62.6 kb
190	17237-L20573	Exon 2	1138-1139	TGACAGAACGAT-AGCAGAAGAACG	1.2 kb
391	17256-L20592	Exon 3	1305-1306	GTGGAAGACGTT-TTTCTTCACTGT	0.8 kb
202	17239-L20575	Exon 4	1429-1430	TGTACGAGCTAT-TCTACCTTACAC	4.7 kb
436	22037-L30961	Exon 5	1538-1539	GTTACAAATTTA-AGAACAGATGAA	3.3 kb
166	17234-L20570	Exon 6	1601-1602	CGGGAAGAAGAT-CCACATGAAGGA	5.4 kb
160	22092-L21513	Exon 7	1644-1645	AGATTTCCAAAC-AGGAAGCTTATA	2.5 kb
356	17253-L20589	Exon 8	1690-1691	AGTCTGCAGTTT-TCTTGTGAGGCC	4.0 kb
275	17246-L21515	Exon 9	1867-1868	ACAGATTGTTGA-AGGATATTATCT	9.4 kb
328	17251-L20587	Exon 10	1934-1935	GACACAGTGGAT-GGCAAGGAAATC	0.9 kb
472	22066-L20571	Exon 11	2124-2125	AACCAAAAGGAT-TAATAGATCTCA	6.4 kb
240	17243-L31376	Exon 12	2214-2215	TTCAGCACTTTA-GTGAAGAACATT	2.3 kb
196 Ж	17238-SP0462-L20574	Exon 13	2299-2300; 2329-2330	GGCATTTTGCAA-30 nt spanning oligo-TAAACGCCTTCG	2.1 kb
285	17247-L31377	Exon 14	2465-2466	CATGCAAGGGAA-GGGCAAAACCCA	0.6 kb
364	17254-L20590	Exon 15	2520-2521	TTCCTCCTGACA-TCAATAGATTTG	1.5 kb
215	17416-L21135	Exon 16	40 nt before exon 16	CTAATTATCGTG-TTCTCTTTTTAA	0.6 kb
142	17231-L20567	Exon 17	2833-2834	ACTGGCCAGCAT-CCTACTGAGGAT	1.5 kb
384	17255-L20591	Exon 18	2972-2973	ATGGAGCAGTAT-ATGAAAGCCACT	1.4 kb
346	17252-L20588	Exon 19	3145-3146	TGTGGAGAAAAT-ATTCATGGCTTC	0.7 kb
481 Ж	22091-SP0463-L21514	Exon 20	3203-3204; 3239-3240	GGGTGTTTACAG-36 nt spanning oligo-ATGAGAACAAGA	3.2 kb
402	17257-L20593	Exon 21	3324-3325	TCAATATCATCT-CAGGTAATCAGC	1.5 kb
319	17250-L20586	Exon 22	3342-3343	CTCCATCTCCTA-TTGCTGCAAGAA	1.5 kb
178	17236-L20572	Exon 23	3444-3445	GTGTCAATCCAT-TCATCAAAAGCA	2.6 kb
256	17245-L20581	Exon 24	3590-3591	GCTCATTCAGAT-GAACTTCGAACG	1.4 kb
154	17233-L20569	Exon 25	3761-3762	GTCCAACATGGT-AATTCACTTCAG	
		stop codon	*3711-3713 (Exon 25)*		

[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.

Table 2b. *EPHB4*

Length (nt)	SALSA MLPA probe	*EPHB4* exon[a]	Ligation site NM_004444.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*494-496 (Exon 1)*		
184 «	22096-L31083	Exon 1	494-495	GAGGCGGCGCCA-TGGAGCTCCGGG	2.8 kb
136 «	22095-L31420	Exon 2	597-596, reverse	CCACCTGAGGGA-ATGTCACCCACT	0.3 kb
334 «	22104-L31091	Exon 3	658-659	CAGCACAGCGTG-CGCACCTACGAA	1.6 kb
418 «	22106-L31093	Exon 4	1270-1269, reverse	TCAGCTGCCTCG-AACCCCGGAGCA	2.1 kb
292 «	22103-L31090	Exon 5	1382-1383	ACTCTAACACCA-TTGGATCAGCCG	0.6 kb
232 «	22099-L31086	Exon 6	1691-1692	TACGTCCTGACT-TCACCTATACCT	1.0 kb
261 «	22101-L31378	Exon 7	1827-1826, reverse	TGCTGGGTGAGG-ACCGCGTCACCC	1.3 kb
409 «	22105-L31092	Exon 8	2006-2005, reverse	CCGTACCTGCAC-CAGGTAGCTGGC	3.3 kb
463	22109-L31096	Exon 9	2172-2173	TGTGGTCGCAGT-TCTCTGCCTCAG	0.7 kb
	No probe	Exon 10			
427	22107-L31094	Exon 11	2257-2258	TCAGGTACTAAG-GTCTACATCGAC	0.3 kb
493	22110-L31097	Exon 12	2428-2429	AGCTGTGTGGCA-ATCAAGACCCTG	5.5 kb
454	22108-L31095	Exon 13	2782-2783	GGCCTTTCCCGA-TTCCTGGAGGAG	0.8 kb
268	22102-L31089	Exon 14	2830-2831	TTTCTGCAGGGA-GGAAAGATTCCC	0.9 kb
220	22097-L31084	Exon 15	2983-2984	TTCCAGGTGATC-AATGCCATTGAA	0.4 kb
130	22094-L31081	Exon 16	3248-3249	GGCTTCGGGCCA-TCAAAATGGGAA	1.7 kb
226	22098-L31085	Exon 17	3381-3382	GAAAATCTTGGC-CAGTGTCCAGCA	
		stop codon	*3455-3457 (Exon 17)*		

[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.

« 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.

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

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Selected publications using SALSA MLPA Probemix P409 RASA1-EPHB4

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- Saliou G et al. (2017). Clinical and genetic findings in children with central nervous system arteriovenous fistulas. *Ann Neurol*. 82(6):972-980.
- Wooderchak-Donahue WL et al. (2018). Expanding the clinical and molecular findings in RASA1 capillary malformation-arteriovenous malformation. *Eur J Hum Genet*. 26(10):1521-1536.

P409 product history	
Version	*Modification*
B1	Probes for *EPHB4* have been included. One probe for *RASA1* exon 4 and one reference probe have been removed. Eight reference probes have been replaced and several probe lengths have been adjusted.
A1	First release.

Implemented changes in the product description
Version B1-02 – 20 September 2022 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *RASA1* and *EPHB4* genes updated according to new versions of the NM_ reference sequences. - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. Version B1-01 – 30 November 2018 (01P) - Product description restructured and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).

More information: www.mrcholland.com; www.mrcholland.eu	
	MRC Holland bv; Willem Schoutenstraat 1 1057 DL, Amsterdam, The Netherlands
E-mail	info@mrcholland.com (information & technical questions) order@mrcholland.com (orders)
Phone	+31 888 657 200