Product Description
SALSA® MLPA® Probemix P155-E1 EDS

To be used with the MLPA General Protocol.

Version E1
As compared to version D2, ten new target probes have been included and one flanking probe was removed. Five reference probes have been replaced and one reference probe was removed. Lengths of ten probes are adjusted. For complete product history see page 12.

Catalogue numbers:
- **P155-025R:** SALSA MLPA Probemix P155 EDS, 25 reactions.
- **P155-050R:** SALSA MLPA Probemix P155 EDS, 50 reactions.
- **P155-100R:** SALSA MLPA Probemix P155 EDS, 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.

Intended purpose
The SALSA MLPA probemix P155 EDS is an in vitro diagnostic (IVD)[1] or a research use only (RUO) semi-quantitive assay[2] for the detection of deletions or duplications in the *COL3A1* and *TNXB* genes in genomic DNA isolated from human peripheral blood specimens in order to confirm a potential cause for and clinical diagnosis of vascular Ehlers-Danlos syndrome (vEDS) and classic-like Ehlers-Danlos syndrome (clEDS), respectively. This product can also be used for molecular genetic testing of at-risk family members. Of note, the majority of copy number variations of *TNXB* are fusions with the *TNXA* pseudogene that include loss of the *CYP21A2* gene, resulting in the contiguous gene syndrome disorder CAH-X.

Copy number variations (CNVs) detected with P155 EDS should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in the *COL3A1* and *TNXB* genes are point mutations, none of which will be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis.

Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation or prenatal testing, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1]Please note that this probemix is for in vitro diagnostic (IVD) use in the countries specified at the end of this product description. In all other countries, the product is for research use only (RUO).

[2]To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

Clinical background

Ehlers-Danlos syndrome (EDS) is a group of disorders affecting the connective tissues that support the skin, bones, blood vessels, and many other organs and tissues. The defects that arise in the connective tissue result in the specific symptoms associated with EDS and range from mildly loose joints to life-threatening complications. Initially the different types of EDS were named with roman numerals (EDS I, EDS II, etc.), but in 1997 the Villefrance nosology for the different EDS types was proposed (Beighton *et al*. 1998), which was revised in 2017 (Malfait *et al*. 2017). The 2017 International Classification of the Ehlers–Danlos Syndromes has become the generally accepted standard and lists thirteen types, with descriptive names such as classic EDS (cEDS), classic-like EDS (clEDS) and vascular EDS (vEDS), which is also used here.

Vascular EDS is an autosomal dominant disorder solely associated with mutations in the *COL3A1* gene. Patients have arterial, intestinal and/or uterine fragility; thin translucent skin that bruises easily; characteristic facial appearance (thin vermilion of the lips, micrognathia, narrow nose, prominent eyes); and an aged appearance to the extremities, particularly the hands. vEDS is a severe disorder: the most prominent signs in patients are vascular dissection or rupture, gastrointestinal perforation, or organ rupture. Approximately 25% of patients experience a severe complication before they reach 20 years of age, and at 40 years ~80% have severe complications. The median age of death is estimated at 50 years. Estimates of the prevalence range from 1:50,000 to 1:200,000. In >95% of patients a *COL3A1* mutation can be identified, and >95% of the known *COL3A1* mutations are point mutations or small indels that are detected with sequence analysis. 1-2% of the known pathogenic mutations in *COL3A1* are large deletions or duplications that can be detected by MLPA (Pepin *et al*. 2014). More information about vEDS can be found at: https://www.ncbi.nlm.nih.gov/books/NBK1494/ and https://omim.org/entry/130050.

Classic-like EDS is an autosomal recessive disorder associated with mutations in the *TNXB* gene. clEDS is primarily characterised by hyperextensible skin, generalised joint hypermobility, muscle weakness, intestinal & tracheal tissue weakness, and in some cases mitral valve prolapse, which affects blood flow between the chambers of the heart. clEDS is very rare, but all known patients have mutations in *TNXB*. Determining the *TNXB* genotype can be challenging because the gene is located in a complex genomic locus that contains a tandem duplicated region (Fig. 1; based on Gitelman *et al.* 1992). This cluster of genes (*C4*, *CYP21* and *TNX*) is also referred to as RCCX module.

TNXB genotyping difficulties are mainly due to the presence of a pseudogene (*TNXA*), which is almost completely identical to the 3'-end of *TNXB* (exons 32–44). The only exception is a 120 basepair (bp) *TNXB*-specific sequence in exon 35. Currently, it is not possible to specifically detect *TNXB* CNVs in the highly homologous exons 32–34 and 36–44. CNV analysis of exon 35 and of sequences upstream of exon 32 and downstream of exon 44 are used to infer whether deletions or duplications of the highly homologous exons are present. Based on recent research the relative frequency of CNVs among pathogenic *TNXB* mutations is estimated at ~30%, most of which originate from rearrangements between *TNXB* and *TNXA* that include the common 120 bp deletion in exon 35, also described as the c.11435_11524+30 deletion (Demirdas *et al*. 2017, Green *et al*. 2020).

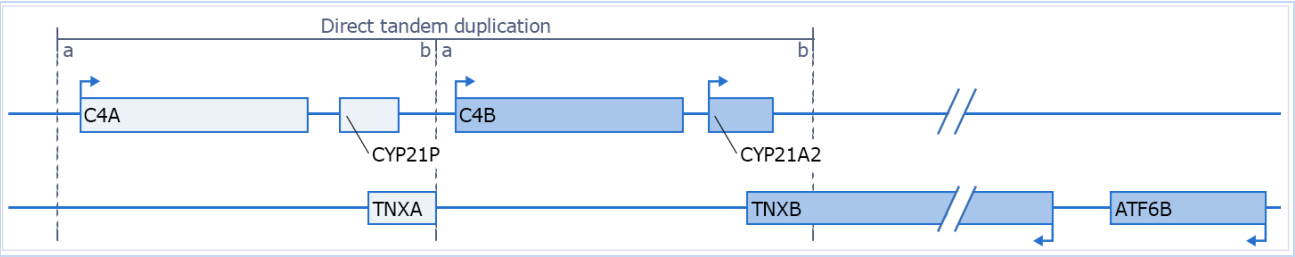

Figure 1. A schematic representation of ~150 kb chromosomal region with the duplicated gene cluster containing the *TNXA* & *CYP21A1P* pseudogenes (light) and the *TNXB* & *CYP21A2* genes (dark), as it is present in the reference genome. Arrows indicate direction of transcription. Note that the 3'-ends of *CYP21A2* and *TNXB* overlap. The figure represents genomic position chr6:31,946,000-32,097,000 of the GRCh37/hg19 assembly. The figure is based on Gitelman *et al.* 1992.

Unequal crossovers between the tandem duplicated regions occur frequently and result in chimeric pseudogene-gene products. Bimodular RCCX - as depicted in Figure 1 - has an allele frequency of ~69% and is considered the 'wild type' situation, but monomodular and trimodular RCCX structures are also frequent and account for ~17% and ~14%, respectively (Blanchong *et al.* 2000). In addition, due to the high homology between *CYP21A2* and *CYP21A1P*, but also between *TNXB* and *TNXA,* gene conversions are likely to occur in these regions. When the functional gene obtains sequence(s) form the non-functional pseudogene or *vice versa* this is referred to as a gene conversion. Gene conversions can present as apparent CNVs and can be benign, especially when the pseudogene obtains sequences from the functional gene.

When unequal crossover occurs between *CYP21A1P* and *CYP21A2,* it will result in a non-transcribed chimeric gene fusion (*CYP21A1P-CYP21A2*). When both alleles of *CYP21A2* are lost or non-functional this leads to the disease Congenital Adrenal Hyperplasia (CAH). **The P155 probemix is not suitable to detect all known *CYP21A1P-CYP21A2* gene fusions. To determine the copy number of *CYP21A2* and detect more *CYP21A1P-CYP21A2* fusion genes the SALSA MLPA Probemix P050 CAH is recommended.** An unequal crossover between *CYP21A1P* and *CYP21A2* does not affect the *TNXB* gene. However, ~10% of CAH patients also have *TNXB* deficiency. This contiguous gene syndrome is referred to as CAH-X. In all known cases this is the result of unequal crossover between *TNXA* and *TNXB*, resulting in a non-functional *TNXB-TNXA* chimeric fusion gene and deletion of the *CYP21A2* gene that is located amidst *TNXA* and *TNXB*. The known *TNXA-TNXB* fusions described in literature (Burch *et al*. 1997; Morissette *et al*. 2015) can be detected by the SALSA MLPA Probemix P155 EDS. More information about clEDS can be found at: https://omim.org/entry/606408.

Gene structure
The *COL3A1* gene spans ~38 kilobases (kb) on chromosome 2q32.2 and contains 51 exons. The *COL3A1* LRG_3 is available at www.lrg-sequence.org and is identical to GenBank NG_007404.1, except for a single mismatch.

The *TNXB* gene is located on chromosome 6p21.32 (reverse strand), spans ~68 kb and contains 44 exons. No LRG is available for *TNXB*, but the GenBank chromosomal sequence is NG_008337.2. *TNXB* is localized to the major histocompatibility complex (MHC) class III region on chromosome 6, and *TNXB* exons 32-44 are part of a duplicated cluster of genes. The duplicated section of *TNXB* is referred to as the *TNXA* pseudogene. In addition, the 3' end of *TNXB* overlaps with the 3' end of *CYP21A2* (Fig. 1).

Transcript variants
For *COL3A1,* one transcript variant has been described encoding the full length protein (NM_000090.4; 5490 nucleotides (nt); coding sequence (CDS) 118-4518; https://www.ncbi.nlm.nih.gov/gene/1281). The ATG translation start site is located in exon 1 and the stop codon is located in exon 51.

For *TNXB,* three variants have been described. Transcript variant XB encodes isoform 1 and is a reference standard in the RefSeqGene project (NM_019105.8; 13091 nt; CDS 167-12895; https://www.ncbi.nlm.nih.gov/gene/7148). The ATG translation start site is located in exon 2 and the stop codon is located in exon 44.

Exon numbering
The *COL3A1* exon numbering used in this P155-E1 EDS product description is the exon numbering from the LRG_3 sequence. 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.

The *TNXB* exon numbering used in this P155-E1 EDS product description is the exon numbering from the NG_008337.2 sequence. 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 P155-E1 EDS contains 54 MLPA probes with amplification products between 124 and 504 nt. This includes 26 probes for the *COL3A1* gene and 20 probes for the *TNXB* gene region. 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 108 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 from human peripheral blood, 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 EDS. 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. Sample ID numbers NA03918, NA10401 and NA11213 from the Coriell Institute have been tested with this P155-E1 probemix at MRC Holland and can be used as positive control samples to detect the CNVs listed in the table below. The quality of cell lines can change; therefore samples should be validated before use.

Sample ID Coriell biobank	Genotype and affected probes	Expected final ratio
NA03918	Heterozygous deletion of *COL3A1* affecting all *COL3A1* probes	0.5
NA10401	Heterozygous duplication of *COL3A1* affecting all *COL3A1* probes	1.5
NA11213	Heterozygous deletion of *COL3A1* affecting all *COL3A1* probes, and heterozygous duplication of *TNXB* exon 35 up until *CYP21A2* affecting *TNXB* exon 35 probes and *CYP21A2* probes	*COL3A1* probes: 0.5 *TNXB* exon 35 & *CYP21A2* probes: 1.5

Performance characteristics

Approximately 1-2% of all vEDS patients have a deletion or duplication in COL3A1 (Pepin *et al.* 2014) and approximately 50% of clEDS have one or two TNXB CNVs (Demirdas *et al.* 2017; Green *et al.* 2020; Gao *et al.* 2021). Most known CNVs of *TNXB* are all rearrangements with the *TNXA* pseudogene leading to deletions of the sequence in between- including the *CYP21A2* gene - and thereby causing the contiguous gene syndrome CAH-X when both alleles are affected. Analytical performance for the detection of deletions/duplications in *COL3A1* and *TNXB* is very high and can be considered >99% (based on a 2010-2021 literature review).

Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

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 expected results for all target probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 3 (heterozygous duplication), 0 (homozygous deletion) or 4 (homozygous duplication or heterozygous triplication). 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. 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.

P155 specific notes

- Due to the high homology between *TNXB* exon 32-34 and 36-44 and the *TNXA* pseudogene, no probes can be designed that are specific for these exons. The two probes targeting *CYP21A2* are included to assist in the detection of *TNXB* deletions or duplications downstream of exon 35.
- The P155 probemix is not suitable to detect all known *CYP21A1P-CYP21A2* gene fusions. To determine the copy number of *CYP21A2* and detect more *CYP21A1P-CYP21A2* fusion genes the SALSA MLPA Probemix P050 CAH is recommended.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *COL3A1* and *TNXB* genes are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P155 EDS.
- 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.

***COL3A1* mutation database**

The LOVD page of *COL3A1* can be found at: https://databases.lovd.nl/shared/genes/COL3A1. We strongly encourage users to deposit positive results in the LOVD database.

***TNXB* mutation database**

The LOVD page for *TNXB* can be found at: https://databases.lovd.nl/shared/genes/TNXB. We strongly encourage users to deposit positive results in the LOVD database.

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 *TNXB* exons 14 and 16 but not exon 15 to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P155-E1 EDS

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*COL3A1*	*TNXB* region
64-105	Control fragments – see table in probemix content section for more information			
124 ¥	Reference probe 18709-L21056	5q		
131 *	**COL3A1** probe 22225-L31330		**Exon 41**	
136	**COL3A1** probe 17516-L21415		**Exon 23**	
142	**TNXB** probe 05007-L04393			**Exon 1**
148	**COL3A1** probe 05018-L04404		**Exon 1**	
154 +๏	**TNXB** probe 13291-L14636			**Exon 35**
161	**COL3A1** probe 17517-L21416		**Exon 14**	
166 *	Reference probe 05721-L31493	7q		
172	**TNXB** probe 05008-L15001			**Exon 3**
178	**COL3A1** probe 05021-L04407		**Exon 5**	
185 ¬∞๏	**CYP21A2** probe 15221-L20260			Downstream
190	**TNXB** probe 05009-L22188			**Exon 5**
196	**COL3A1** probe 05019-L04405		**Exon 1**	
202	Reference probe 08187-L08081	11q		
208 ¥	**TNXB** probe 05012-L31728			**Exon 12**
215	**TNXB** probe 17518-L21417			**Exon 2**
221	**COL3A1** probe 05020-L21329		**Exon 2**	
226	**COL3A1** probe 20544-L28408		**Exon 47**	
232 ¬∞๏	**CYP21A2** probe 15216-L16990			Downstream
238	**TNXB** probe 05010-L04396			**Exon 8**
244 *	Reference probe 21641-L30683	6q		
250 *	**COL3A1** probe 22230-L31335		**Exon 16**	
256 ¥	**COL3A1** probe 05223-L31492		**Exon 43**	
260 ¥	**COL3A1** probe 20545-L31302		**Exon 36**	
265 *	**COL3A1** probe 22229-L31334		**Exon 22**	
274	**COL3A1** probe 05022-L21402		**Exon 11**	
283 ∫	**TNXB** probe 05011-L04397			**Exon 11**
288 *	**COL3A1** probe 22228-L31333		**Exon 27**	

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*COL3A1*	*TNXB* region
296 ¥	**TNXB** probe 01982-L31303			**Exon 31**
303 *	Reference probe 15461-L17667	17q		
310 ¥	**TNXB** probe 03033-L31304			**Exon 14**
319	**COL3A1** probe 17521-L21420		**Exon 9**	
326	**TNXB** probe 01980-L21389			**Exon 1**
333 *	**COL3A1** probe 22223-L31895		**Exon 49**	
340 ¥¬	**ATF6B** probe 01979-L31305			Upstream
350 *	**COL3A1** probe 22227-L31332		**Exon 34**	
358	Reference probe 16442-L18895	18q		
364	**COL3A1** probe 05024-L04410		**Exon 20**	
372	**COL3A1** probe 17522-L21421		**Exon 4**	
382	**TNXB** probe 05013-L14827			**Exon 15**
391 ±	**TNXB** probe 05015-L04401			**Exon 23**
400	**COL3A1** probe 05023-L04409		**Exon 17**	
409 *	**COL3A1** probe 22224-L31329		**Exon 45**	
418	**TNXB** probe 05016-L15002			**Exon 26**
427	**COL3A1** probe 17523-L21422		**Exon 28**	
436	**TNXB** probe 17524-L21423			**Exon 16**
444 *	Reference probe 09077-L23425	19p		
452 *	**COL3A1** probe 05026-L23956		**Exon 32**	
461 ¥	**TNXB** probe 05014-L31308			**Exon 19**
471 *	**COL3A1** probe 22231-L31336		**Exon 7**	
477 ¥+ꝏ	**TNXB** probe 13292-L31309			**Exon 35**
486 ¥	**COL3A1** probe 05028-L31311		**Exon 51**	
494 *	**COL3A1** probe 22360-L31519		**Exon 39**	
504 *	Reference probe 09870-L19465	2p		

[a] See section Exon numbering on page 3 for more information.

* New in version E1.

¥ Changed in version E1. Minor alteration, no change in sequence detected.

∞ Wild type sequence detected. These probes detect *CYP21A2* specific sequences. Due to the high homology of *CYP21A2* with the *CYP21A1P* pseudogene, gene conversions can occur. Gene conversions can be detected as apparent deletions or duplications and are not always pathogenic. The 185 nt probe signal is decreased in the presence of the 8 bp deletion *CYP21A2* c.332_339del. The 232 nt probe signal is decreased in the presence of the *CYP21A2* V237E mutation and to a lesser extent in the presence of the *CYP21A2* M240K.

± SNP rs369180703 can influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

+ The 154 nt and 477 nt probes are within the 120 bp sequence that is absent in the *TNXA* pseudogene and can therefore be used to detect the frequent 120 bp deletion *TNXB* c.11435_11524+30 deletion (exon 35).

ꝏ Copy number variations are frequent in the general population.

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

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 2. P155-E1 probes arranged according to chromosomal location

Table 2a. *COL3A1*

Length (nt)	SALSA MLPA probe	*COL3A1* exon[a]	Ligation site NM_000090.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
196	05019-L04405	Exon 1	71-72	TTGAACTGCTTT-TCTTTTCTCCTT	0.1 kb
		start codon	*118-120 (exon 1)*		
148	05018-L04404	Exon 1	177-178	CATCCCACTATT-ATTTTGGCACAA	10.3 kb
221	05020-L21329	Exon 2	314-315	CGATGACATAAT-ATGTGACGATCA	0.9 kb
372	17522-L21421	Exon 4	540-541	CCTGGAATCTGT-GAATCATGCCCT	1.4 kb
178	05021-L04407	Exon 5	633-634	CTCGCAGGCTAT-CCTGGACCAGCT	1.5 kb
471	22231-L31336	Exon 7	751-752	AAGCTGGTCCTT-CAGTAAGTAACA	1.5 kb
319	17521-L21420	Exon 9	829-830	GTAGACCCGGAC-GACCTGGAGAGC	0.9 kb
274	05022-L21402	Exon 11	939-938 reverse	GTTTCACCCTTT-TCTCCATTTCGT	1.2 kb
161	17517-L21416	Exon 14	1081-1082	GTGCTCGGGGTA-ATGACGGTGCTC	1.2 kb
250	22230-L31335	Exon 16	1173-1172 reverse	GCAGGTCCAACT-TCACCCTAATGG	0.7 kb
400	05023-L04409	Exon 17	4 nt after exon 17	GCGAAATGGTAA-GCTGTCCCCACT	0.7 kb
364	05024-L04410	Exon 20	1494-1495	GGTGTTCCAGGA-GCTAAAGGCGAA	0.8 kb
265	22229-L31334	Exon 22	99 nt before exon 22, reverse	CATTTGAACTTT-TAAAAAGGGTTC	0.5 kb
136	17516-L21415	Exon 23	1729-1730	TACTTCAGGGCA-TGCCCGGAAGTC	1.6 kb
288	22228-L31333	Exon 27	2035-2034 reverse	TCTTACTTGTAA-TCCTTGTGGACC	0.6 kb
427	17523-L21422	Exon 28	2070-2071	GGTCCTCCAGGA-GAAAATGGAAAA	1.6 kb
452	05026-L23956	Exon 32	2398-2399	AAGATGGCCCAA-GGGTGAGTATTC	1.7 kb
350	22227-L31332	Exon 34	2502-2503	GGACCTCGTGGT-AGCCCTGTAAGT	1.4 kb
260	20545-L31302	Exon 36	2575-2576	GACAGAATGGTG-AACCTGGTGGTA	1.2 kb
494	22360-L31519	Exon 39	2935-2934 reverse	ACTTACTGGTGG-GCCCTGGGCACC	1.2 kb
131	22225-L31330	Exon 41	3063-3064	GAAAGTGGGAAA-CCAGGAGCTAAC	1.1 kb
256	05223-L31492	Exon 43	4 nt after exon 43	GAGAAAGTGTGA-GTTCCCAAAAGC	1.1 kb
409	22224-L31329	Exon 45	3420-3421	GGTGAACGTGGA-GCTGCTGGCATC	0.6 kb
226	20544-L28408	Exon 47	3600-3601	GGTCCCATTGGA-CCACCAGGGCCT	2.2 kb
333	22223-L31895	Exon 49	4039-4040	GTGCCAATCCTT-TGAATGTTCCAC	1.5 kb
486	05028-L31311	Exon 51	4484-4485	TGATCAAGAATT-TGGTGTGGACGT	-
		stop codon	*4516-4518 (exon 51)*		

Table 2b. *TNXB* region

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site NM_019105.8	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
340 ¬	01979-L31305	*ATF6B*		GACAACCTGCTT-AGCCCGGAGGAC	18.6 kb
		TNXB			
142	05007-L04393	Exon 1	202 nt before exon 1	CGTGGGAGGTAA-GACCGGGGCTGG	0.3 kb
326	01980-L21389	Exon 1	112-113	CCTCCCGGGGTT-GGGGACAGAGCA	11.4 kb
		start codon	*167-169 (exon 2)*		
215	17518-L21417	Exon 2	530-531	AGGAACAGTGCA-CTGGGGGATGTT	1.1 kb
172	05008-L15001	Exon 3	1332-1331 reverse	CCGTGTCGCAAA-TGCATTCGCCGT	7.4 kb
190	05009-L22188	Exon 5	2581-2582	GCCTCAGCCTAT-GACCAGAGAGGA	4.7 kb
238	05010-L04396	Exon 8	3402-3403	CGACTCCTTGCT-CCTGCGCTGGAC	5.5 kb
283 ∫	05011-L04397	Exon 11	4456-4457	GTGGGAGGCCTA-GAGCCCGGGCAC	5.2 kb
208	05012-L31728	Exon 12	4627-4626 reverse	CCCACAGAGTTG-GGGGTCACATCT	3.6 kb

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site NM_019105.8	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
310 #	03033-L31304	Exon 14	5289-5290	TGTGGTCCAGTT-CAAGGACAAAGA	0.6 kb
382	05013-L14827	Exon 15	5649-5650	CAGCCTCAGGGA-GGTCAGCGTGCC	0.8 kb
436	17524-L21423	Exon 16	6023-6024	TGTATGGTTTCA-GTGATGGGAAGC	3.8 kb
461	05014-L31308	Exon 19	6745-6746	GCTCCTCTTGCA-AAGCTGCGCCTA	8.3 kb
391 ±#	05015-L04401	Exon 23	8072-8073	GGGAGCTGACCA-TGACAGATGCCA	4.1 kb
418 #	05016-L15002	Exon 26	9262-9261 reverse	GTCACACCCACA-GCGGACACTGGG	6.3 kb
296	01982-L31303	Exon 31	10554-10555	CTCCAGCTCTCT-GCGCCTGTCCTG	2.6 kb
154 +◎	13291-L14636	Exon 35	11608-11609	CAGGGGCTGATC-CCAGGCGCTCGC	0.1 kb
477 +◎	13292-L31309	Exon 35	11670-11669 reverse	GGAAGCCTGTGA-GAGGCTCACTCT	4.0 kb
		stop codon	*12893-12895 (exon 44)*		
232 ∞¬◎#	15216-L16990	*CYP21A2* Exon 6		GGATCACATCGT-GGAGATGCAGCT	0.7 kb
185 ∞¬◎	15221-L20260	*CYP21A2* Exon 3		GGAGACTACTCC-CTGCTCTGGAAA	-

[a] See section Exon numbering on page 3 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.

∞ Wild type sequence detected. These probes detect *CYP21A2* specific sequences. Due to the high homology of *CYP21A2* with the *CYP21A1P* pseudogene, gene conversions can occur. Gene conversions can be detected as apparent deletions or duplications and are not always pathogenic. The 185 nt probe signal is decreased in the presence of the 8 bp deletion *CYP21A2* c.332_339del. The 232 nt probe signal is decreased in the presence of the *CYP21A2* V237E mutation and to a lesser extent in the presence of the *CYP21A2* M240K.

± SNP rs369180703 can influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

+ The 154 nt and 477 nt probes are within the 120 bp sequence that is absent in the *TNXA* pseudogene and can therefore be used to detect the frequent 120 bp deletion *TNXB* c.11435_11524+30 deletion (exon 35).

◎ Copy number variations are frequent in the general population.

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.

∫ It has been experimentally confirmed for this probe that a single nucleotide change in a related sequence can result in a false duplication result; rs61740331 in *TNXB* exon 28 creates a secondary genomic target for this probe resulting in an increased signal. In case of an apparent duplication of only the *TNXB* exon 11 probe (283 nt), it is recommended to sequence the genomic region surrounding rs61740331.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Complete probe sequences are available at www.mrcholland.com.

Related SALSA MLPA probemixes

P050 CAH	Contains probes for the *CYP21A2* gene associated with CAH, probes for the *CYP21A1P* pseudogene, and several probes for the *TNXB* gene.

P272 COL1A2	Contains probes for the *COL1A2* gene, which is associated to two other types of EDS: arthrochalasia EDS and cardiac-valvular EDS, according to the revised Villefrance nosology. These types were formerly known as EDS VIIA and VIIB.
P331/P332 COL5A1	Contain probes for the *COL5A1* gene, which is associated with classical EDS, according to the revised Villefrance nosology. This type was formerly known as EDS I and II.
P359 PLOD1	Contains probes for the *PLOD1* gene, which is associated with kyphoscoliotic EDS, according to the revised Villefrance nosology. This type was formerly known as EDS VI.

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Selected publications using SALSA MLPA Probemix P155 EDS

- Demirdas S *et al*. (2017). Recognizing the tenascin-X deficient type of Ehlers-Danlos syndrome: a cross-sectional study in 17 patients. *Clin Genet*. 91:411-425.
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- Morissette R *et al*. (2015). Broadening the Spectrum of Ehlers Danlos Syndrome in Patients With Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab*. 100:E1143-1152.
- Nayak SS *et al*. (2021). Clinically relevant variants in a large cohort of Indian patients with Marfan syndrome and related disorders identified by next-generation sequencing. *Sci Rep*. 11:764.
- Ritelli M *et al*. (2020). Application of the 2017 criteria for vascular Ehlers-Danlos syndrome in 50 patients ascertained according to the Villefranche nosology. *Clin Genet*. 97:287-295.

P155 product history	
Version	*Modification*
E1	Ten new target probes have been included and one flanking probe has been removed. Five reference probes have been replaced and one reference probe removed. Lengths of ten probes have been adjusted.
D2	One reference probe has been replaced.
D1	One reference probe and one target probe have been removed, and the length of some probes has been adjusted.
C1	The number of target probes has been increased from 26 to 34, the number of reference probes decreased to 10, QDX2 fragments have been added.
B1	Two new *TNXB* probes for exon 35 have been added. Three variable probes for *TNXB* exon 40 and the nearby *C4B* and *CYP21A1P* genes have been removed. In addition, four extra control fragments at 88-96-100-105 nt have been included.
A1	First release.

Implemented changes in the product description

Version E1-04 – 01 March 2023 (04P)
- Header and numbering of table 2 (a+b) corrected.

Version E1-03 – 12 October 2021 (04P)
- Product description rewritten and adapted to a new template.
- *Performance characteristics* updated with diagnostic sensitivity for *TNXB*.
- *COL3A1* mutation database changed.
- Ligation sites of the probes targeting the *COL3A1* gene updated according to new version of the NM_ reference sequence.
- Warning added to 283 nt probe for false duplication results due to a SNP in *TNXB* exon 28 in Table 2.
- Sections *References* and *Selected publications* curated.
- UK has been added to the list of countries in Europe that accept the CE mark.

Version E1-02 – 28 October 2020 (02P)
- Intended use changed to Intended purpose using new template text.
- Section Clinical background of clEDS was rewritten for clarification reasons.
- New P155 specific note added in section Interpretation of results
- Remarks, with symbols ∞ and +, under Table 1 and 2 has been rephrased.
- Updated References and Selected publications using SALSA MLPA Probemix P155 EDS with recent scientific literature.
- Various minor textual or layout changes.

Version E1-01 – 03 October 2019 (02P)
- Product description rewritten and adapted to a new template.
- P155-E1 is now CE marked.
- Ligation sites of the probes targeting the TNXB gene updated according to new version of the NM_019105.8 reference sequence.
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).
- Warning added to Table 1 and 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.

- For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36).
- Figure 1 has been updated.

Version D2-01 – 21 January 2019 (01P)
- Product description restructured and adapted to a new template.
- For uniformity, the chromosomal positions and bands in this document are now all based on hg18 (NCBI36).
- Note added that many samples from healthy individuals show a duplication of CYP21A2 probe signals, in which case the pseudogene most likely has acquired the sequence of the wildtype CYP21A2 gene.
- Symbol added in Table 1 and 2 for flanking probes.
- Updated information of related product P050 CAH.

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

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