# Product Description
## SALSA® MLPA® Probemixes
## P034-B2 DMD-1 & P035-B1 DMD-2

To be used with the MLPA General Protocol.

**P034 version B2**
For complete product history see page 12.
**P035 version B1**
For complete product history see page 12.

**Catalogue numbers:**
- **P034-025R:** SALSA MLPA Probemix P034 DMD-1, 25 reactions.
- **P034-050R:** SALSA MLPA Probemix P034 DMD-1, 50 reactions.
- **P034-100R:** SALSA MLPA Probemix P034 DMD-1, 100 reactions.

- **P035-025R:** SALSA MLPA Probemix P035 DMD-2, 25 reactions.
- **P035-050R:** SALSA MLPA Probemix P035 DMD-2, 50 reactions.
- **P035-100R:** SALSA MLPA Probemix P035 DMD-2, 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 Probemixes P034 DMD-1 and P035 DMD-2 are in vitro diagnostic (IVD)[1] or a research use only (RUO) semi-quantitative assays[2] for the detection of deletions or duplications in the *DMD* gene in genomic DNA isolated from human peripheral whole blood specimens, (un)cultured amniotic fluid obtained in week 16 of the pregnancy or later and free from blood contamination, (un)cultured chorionic villi free from maternal contamination, or fetal blood. P034 DMD-1 and P035 DMD-2 are intended to confirm a potential cause for and clinical diagnosis of Duchenne muscular dystrophy or Becker muscular dystrophy, for molecular genetic testing of at-risk family members, and for carrier screening.

Copy number variations (CNVs) detected with P034 DMD-1 and P035 DMD-2 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 *DMD* gene are CNVs, however point mutations can occur which will not 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, parental evaluation, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

These devices are not intended to be used for standalone diagnostic purposes, pre-implantation, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1] Please note that these probemixes are 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

Germline defects in the dystrophin *(DMD)* gene are the most frequent cause of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). DMD and BMD occur almost exclusively in males as they are inherited in an X-linked recessive manner. DMD usually has an early onset in childhood with delayed milestones, which include delays in sitting and standing independently. Proximal weakness causes a waddling gait and difficulty in climbing. DMD is rapidly progressive, with affected children being wheelchair dependent by the age of 13. Respiratory complications and cardiomyopathy occur in individuals with DMD after the age of 18 and a few survive beyond the third decade of life. In contrast, BMD has a slower rate of progression and patients on average survive until their mid-40s. More information on both conditions is available at http://www.ncbi.nlm.nih.gov/books/NBK1119/.

Deletions and duplications of complete exons in the *DMD* gene are the most frequent cause of DMD/BMD and are usually missed by standard sequence analysis. Most of these deletions and duplications can be detected by the MLPA technique and hence MLPA complements sequence analysis of the *DMD* gene. Approximately 60-70% of mutations found in patients with DMD and BMD are deletions. Duplications in the DMD gene are found in 5-15% of DMD patients and 20% of BMD patients, respectively (Duan et al. 2021). Best practice guidelines on molecular diagnostics in DMD and BMD have been published (Abbs et al. 2010, Fratter et al. 2020).

## Gene structure

The *DMD* gene spans ~2.1 megabases (Mb) (NM_004006.3) on chromosome Xp21.1-p21.2 and contains 79 exons. The *DMD* LRG_199 is available at www.lrg-sequence.org and is identical to GenBank NG_012232.1.

## Transcript variants

For *DMD,* multiple variants have been described. Transcript variant Dp427m encodes the main dystrophin protein found in muscle (NM_004006.3; 13992 nt; coding sequence 238-11295). *DMD* transcript variant Dp427c encodes the main dystrophin protein found in brain (NM_000109.4; 13854 nt; coding sequence 124-11157). This transcript uses an alternative promoter/exon 1, which is located upstream of the Dp427m transcript promoter. The other 78 exons are identical in both transcripts.

## Exon numbering

The *DMD* exon numbering used in this P034-B2 DMD-1 and P035-B1 DMD-2 product description is the exon numbering from the LRG_199 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 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 P034-B2 DMD-1 contains 49 MLPA probes with amplification products between 130 and 500 nucleotides (nt). The SALSA MLPA Probemix P035-B1 DMD-2 contains 48 MLPA probes with amplification products between 130 and 500 nt. The P034-B2 and P035-B1 probemixes together contain one probe for each of the 79 *DMD* exons included in transcript variant Dp427m. In addition, one probe is present in P035-B1 for the alternative promoter/exon 1 found in transcript variant Dp427c. Performing two MLPA reactions, one with P034-B2 and one with P035-B1, is thus sufficient to investigate the copy number of all *DMD* exons. In addition, P034-B2 and P035-B1 contain nine and eight reference probes, respectively, which detect locations on the X-chromosome. 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 of the same sex 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 peripheral whole blood specimens, (un)cultured amniotic fluid obtained in week 16 of the pregnancy or later and free from blood contamination, (un)cultured chorionic villi free from maternal contamination, or fetal 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 DMD or BMD. As all probes in P034 and P035 target the X chromosome the gender of the reference samples used in an experiment is not important. Target probes are first normalised to reference probes within a sample. Therefore, a ratio of 1 corresponds to 1 copy in male samples as they have one X chromosome. 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. NA05117, NA05123, NA23087, NA23094 and NA10283 from the Coriell Institute have been tested P034-B2 and P035-B1 probemixes at MRC Holland and can be used as positive control samples (see table below). The quality of cell lines can change; therefore samples should be validated before use.

| Sample ID Coriell | *DMD* CNVs detected by | CNVs found |
|---|---|---|
| NA05117 (female) | P034 | Heterozygous exon 45 deletion |
| NA23087 (female) | P034/P035 | Heterozygous exon 2-30 duplication |
| NA23094 (female) | P034/P035 | Heterozygous exon 35-43 deletion |
| NA05123 (male) | P034/P035 | Exon 45-62 duplication |
| NA10283 (male) | P035 | Exon 72-79 deletion |

**Performance characteristics**

In most populations, approximately 80% of the mutations found in DMD are deletions/duplications which are detectable with these MLPA probemixes (Duan et al. 2021). The analytical sensitivity and specificity for the detection of deletions/duplications in Duchenne and Becker muscular dystrophy patients (based on a 2010-2021 literature review) is very high and can be considered >99%.

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.

**Note: Slope correction in samples with large deletions**

The slope correction algorithm in Coffalyser.Net has been optimised to give the best possible results in as many situations as possible. However, the slope correction algorithm may confuse a large *DMD* deletion and/or a deletion of a sequence targeted by a reference probe for sloping, which can lead to over- or under-correction. Since large deletions are often detected when using the P034/P035 probemixes, this issue may occur relatively frequently. Incorrectly applied slope correction can cause an FSLP warning in Coffalyser.Net or ambiguous results for multiple probes. If you suspect that slope correction was incorrectly applied, we recommend contacting info@mrcholland.com for assistance.

**Interpretation of results**

The expected results for the *DMD* region specific MLPA probes in female DNA samples are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 3 (heterozygous duplication) and occasionally 4 (heterozygous triplication). For male DNA samples, copy numbers of 1 (normal), 0 (deletion), or 2 (duplication) can be expected. 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:

| Copy Number status: Male samples | Final ratio |
|---|---|
| Normal | 0.80 < FR < 1.20 |
| Deletion | FR = 0 |
| Duplication | 1.65 < FR < 2.25 |
| Ambiguous copy number | All other values |

| Copy Number status: Female samples | Final ratio |
|---|---|
| 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 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.

P034/P035 specific notes:
- DMD/BMD is an X-linked recessive disorder. In males, alteration of the single *DMD* copy due to a mutation, deletion or partial duplication is therefore sufficient to cause the condition. In females, inactivation of a single copy of the gene is not expected to be pathogenic.
- A deletion of one or more *DMD* exons present in the major transcript variant Dp427m is expected to result in Duchenne or Becker muscular dystrophy in males. Deletion of the promoter/exon 1 of this transcript variant, which is the most abundant transcript in muscle, has been reported to result in X-linked dilated cardiomyopathy (Muntoni et al. 1993). While such a deletion theoretically should preclude muscle-specific transcription, relatively high levels of dystrophin were still detected. The authors suggest that transcription was driven by the alternative brain promoter, present in exon 1 of transcript Dp427c and not in Dp427m, leading to a different phenotype.
- A duplication of an internal part of a gene usually results in a defective copy of that gene, as the duplicated sequence is typically located directly adjacent to the original sequence, resulting in a defective transcript. Duplication of the complete *DMD* gene is not expected to result in disease. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might in some cases not result in inactivation of that gene copy.
- Please note that Schwartz et al. (2007) have reported a completely healthy adult male with a deletion of exon 16 and part of introns 15 and 16. Their findings suggest that some gene rearrangements of the dystrophin gene may not always be disease-causing. Please be cautious with the diagnosis of dystrophinopathy in cases of single exon in-frame deletions.
- Given the above-mentioned findings, one should be cautious with the prediction of an expected phenotype based on genotype. Factors like in-frame/out of-frame, extent and location of mutations in the *DMD* gene have different influences on the phenotype. The http://www.dmd.nl/ website has a tool to predict the

effect of the deletions/duplications of exons on the reading frame. We recommend confirmation on RNA level, as the data derived from the DNA analysis does not provide absolute certainty on the reading frame effect.

**Limitations of the procedure**
- The SALSA MLPA probemixes P034 DMD-1 and P035 DMD-2 will not detect point mutations in the *DMD* gene, which are the second most common cause of genetic defects in the *DMD* gene after CNVs.
- 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.
- For use on (un)cultured amniocytes, contamination of the sample with maternal DNA may lead to wrong conclusions.
- For use on (un)cultured chorionic villi, discrepancies in chromosomal patterns between DNA from chorionic villi and foetus have been described due to maternal contamination, postzygotic nondisjunction, postzygotic isochromosome formation, mosaic situations, and complications in DNA sampling in twin pregnancies (Van den Berg et al. 2006).
- 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 kilobases (kb) in length can often be confirmed by FISH.

***DMD* mutation database**
http://www.dmd.nl/. We strongly encourage users to deposit positive results in the *DMD* Mutation 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 *DMD* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

## Table 1a. SALSA MLPA Probemixes P034-B2 DMD-1

| Length (nt) | SALSA MLPA probe | Chromosomal position (hg18)[a] | |
|---|---|---|---|
| | | Reference | *DMD* |
| 64-105 | Control fragments − see table in probemix content section for more information | | |
| 130 | Reference probe 13499-L02104 | Xp11 | |
| 142 Δ | **DMD probe 01353-L25384** | | **Exon 1 of Dp427m** |
| 149 | **DMD probe 18831-L25385** | | **Exon 41** |
| 157 | **DMD probe 01355-L25386** | | **Exon 21** |
| 165 | **DMD probe 01356-L25387** | | **Exon 61** |
| 172 | **DMD probe 01357-L25388** | | **Exon 2** |
| 178 | Reference probe 07655-L07361 | Xp11 | |
| 187 | **DMD probe 01711-L25389** | | **Exon 42** |
| 193 | **DMD probe 18998-L24802** | | **Exon 22** |
| 199 | **DMD probe 01897-L25390** | | **Exon 62** |
| 205 | Reference probe 07672-L26039 | Xp22 | |
| 213 | **DMD probe 01361-L25391** | | **Exon 3** |
| 220 | **DMD probe 18221-L25392** | | **Exon 43** |
| 226 | **DMD probe 19138-L25106** | | **Exon 23** |
| 231 | Reference probe 00821-L09487 | Xp22 | |
| 241 | **DMD probe 01364-L25393** | | **Exon 63** |
| 247 | **DMD probe 01365-L25394** | | **Exon 4** |
| 254 | **DMD probe 01366-L25396** | | **Exon 44** |
| 262 Δ | **DMD probe 01958-L25397** | | **Exon 24** |
| 268 | **DMD probe 01368-L25398** | | **Exon 64** |
| 278 | Reference probe 05893-L08952 | Xq22 | |
| 284 | **DMD probe 01954-L25724** | | **Exon 5** |
| 291 | **DMD probe 01370-L01287** | | **Exon 45** |
| 303 | **DMD probe 01371-L25399** | | **Exon 25** |
| 312 | **DMD probe 19002-L26038** | | **Exon 65** |
| 319 | **DMD probe 01373-L25725** | | **Exon 6** |
| 325 | **DMD probe 01374-L25401** | | **Exon 46** |
| 332 | **DMD probe 01375-L25402** | | **Exon 26** |
| 341 | **DMD probe 01376-L25403** | | **Exon 66** |
| 346 | Reference probe 03149-L14468 | Xq21 | |
| 357 | **DMD probe 01713-L25404** | | **Exon 7** |
| 364 | **DMD probe 01378-L25405** | | **Exon 47** |
| 373 | **DMD probe 01379-L25406** | | **Exon 27** |
| 381 | **DMD probe 01960-L25407** | | **Exon 67** |
| 391 ± | **DMD probe 19004-L24808** | | **Exon 8** |
| 398 ± | **DMD probe 01382-L25408** | | **Exon 48** |
| 405 | **DMD probe 01716-L25410** | | **Exon 28** |
| 413 | **DMD probe 02482-L28035** | | **Exon 68** |
| 420 | Reference probe 00820-L25090 | Xq26 | |
| 427 | **DMD probe 01385-L25412** | | **Exon 9** |
| 436 | **DMD probe 19006-L24810** | | **Exon 49** |
| 445 | **DMD probe 01387-L25413** | | **Exon 29** |
| 454 | **DMD probe 19341-L25594** | | **Exon 69** |
| 461 | Reference probe 05632-L26218 | Xq28 | |
| 469 | **DMD probe 01718-L26101** | | **Exon 10** |
| 476 | **DMD probe 19340-L25774** | | **Exon 50** |
| 483 | **DMD probe 19008-L24812** | | **Exon 30** |
| 493 | **DMD probe 01392-L27986** | | **Exon 70** |
| 500 | Reference probe 10764-L25900 | Xq23 | |

## Table 1b. SALSA MLPA Probemixes P035-B1 DMD-2

| Length (nt) | SALSA MLPA probe | Chromosomal position (hg18)ᵃ | |
| --- | --- | --- | --- |
| | | Reference | *DMD* |
| 64-105 | Control fragments − see table in probemix content section for more information | | |
| 130 | Reference probe 13498-L06679 | Xp22 | |
| 141 | **DMD probe** 01393-L25414 | | **Exon 11** |
| 148 | **DMD probe** 01394-L25415 | | **Exon 51** |
| 157 | **DMD probe** 01395-L25416 | | **Exon 31** |
| 166 Ж | **DMD probe** 18996-SP0733-L25739 | | **Exon 71** |
| 171 Ж | **DMD probe** 18997-SP0734-L25740 | | **Exon 12** |
| 177 | Reference probe 03110-L22383 | Xq21 | |
| 187 | **DMD probe** 02059-L25417 | | **Exon 52** |
| 193 | **DMD probe** 18999-L24803 | | **Exon 32** |
| 199 | **DMD probe** 01949-L25418 | | **Exon 72** |
| 211 | **DMD probe** 19001-L24805 | | **Exon 13** |
| 219 | **DMD probe** 01892-L01050 | | **Exon 53** |
| 226 | **DMD probe** 19140-L25108 | | **Exon 33** |
| 232 | Reference probe 07669-L07375 | Xp22 | |
| 239 | **DMD probe** 01893-L25419 | | **Exon 73** |
| 247 | **DMD probe** 19338-L25591 | | **Exon 14** |
| 254 | **DMD probe** 01894-L25421 | | **Exon 54** |
| 263 | **DMD probe** 01901-L25422 | | **Exon 34** |
| 269 | **DMD probe** 01902-L25423 | | **Exon 74** |
| 276 | Reference probe 02900-L26167 | Xq26 | |
| 283 | **DMD probe** 19141-L25730 | | **Exon 15** |
| 291 | **DMD probe** 01411-L01058 | | **Exon 55** |
| 297 | Reference probe 06476-L26037 | Xp22 | |
| 303 | **DMD probe** 15720-L25424 | | **Exon 35** |
| 310 | **DMD probe** 01413-L25425 | | **Exon 75** |
| 319 | **DMD probe** 02060-L25426 | | **Exon 16** |
| 325 Ж | **DMD probe** 19003-SP0735-L24807 | | **Exon 56** |
| 332 | **DMD probe** 01416-L25427 | | **Exon 36** |
| 342 | **DMD probe** 01417-L25428 | | **Exon 76** |
| 350 | Reference probe 08245-L09531 | Xq26 | |
| 358 | **DMD probe** 01952-L25429 | | **Exon 17** |
| 364 | **DMD probe** 01419-L25430 | | **Exon 57** |
| 372 | **DMD probe** 03038-L26168 | | **Exon 37** |
| 381 | **DMD probe** 01421-L25432 | | **Exon 77** |
| 388 | **DMD probe** 01891-L25433 | | **Exon 18** |
| 396 ± | **DMD probe** 01423-L01070 | | **Exon 58** |
| 407 | **DMD probe** 19372-L25772 | | **Exon 38** |
| 413 | **DMD probe** 19342-L25595 | | **Exon 78** |
| 422 | Reference probe 06187-L26204 | Xq13 | |
| 427 | **DMD probe** 19005-L24809 | | **Exon 19** |
| 436 | **DMD probe** 01427-L25436 | | **Exon 59** |
| 445 | **DMD probe** 19007-L24811 | | **Exon 39** |
| 453 | **DMD probe** 19142-L25437 | | **Exon 79** |
| 466 | **DMD probe** 01430-L25438 | | **Exon 20** |
| 472 | **DMD probe** 01431-L25439 | | **Exon 60** |
| 481 Δ | **DMD probe** 19009-L24813 | | **Exon 40** |
| 490 | **DMD probe** 01433-L25440 | | **Exon 1 of Dp427c** |
| 500 | Reference probe 10764-L25900 | Xq23 | |

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

Δ More variable. This probe is sensitive to experimental conditions. Aberrant results should be treated with caution.

± SNP rs398124074 could influence the 396 nt probe signal (P035). SNP rs756370588 could influence the 391 nt probe signal and SNP rs398124041 could influence the 398 nt probe signal (P034). In case of apparent deletions, it is recommended to sequence the regions targeted by these probes.

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. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

## Table 2. DMD probes arranged according to chromosomal location

| Length (nt) P034 / P035 | | SALSA MLPA probe | *DMD* exon[a] | Ligation site NM_004006.3 | <u>Partial</u> sequence[b] (24 nt adjacent to ligation site) | Distance to next probe |
|---|---|---|---|---|---|---|
| | | | *start codon 238-240 (Exon 1)* | | | |
| | 490 | 01433-L25440 | Exon 1 of Dp427c | NM_000109.4; 12-13 | GGCAGTAATAGA-ATGCTTTCAGGA | 127.9 kb |
| 142 Δ | | 01353-L25384 | Exon 1 of Dp427m | 55-56 | TTCCCCCTACAG-GACTCAGATCTG | 191.3 kb |
| 172 | | 01357-L25388 | Exon 2 | 295-296 | TTCAAAAGAAAA-CATTCACAAAAT | 170.4 kb |
| 213 | | 01361-L25391 | Exon 3 | 364-365 | ACCTCTTCAGTG-ACCTACAGGATG | 5.0 kb |
| 247 | | 01365-L25394 | Exon 4 | 464-465 | TGCCCTGAACAA-TGTCAACAAGGC | 21.5 kb |
| 284 | | 01954-L25724 | Exon 5 | 546-547 | GTAGATGGAAAT-CATAAACTGACT | 6.7 kb |
| 319 | | 01373-L25725 | Exon 6 | 647-648 | AACCAACAGTGA-AAAGATTCTCCT | 7.0 kb |
| 357 | | 01713-L25404 | Exon 7 | 794-795 | CTGGAATAGTGT-GGTTTGCCAGCA | 110.5 kb |
| 391 ± | | 19004-L24808 | Exon 8 | 10 nt after exon 8 | AGGTAAAGTGTG-TAAAGGACAGCT | 1.1 kb |
| 427 | | 01385-L25412 | Exon 9 | 1095-1096 | GCACAGGGATAT-GAGAGAACTTCT | 52.8 kb |
| 469 | | 01718-L26101 | Exon 10 | 1224-1225 | GAAGACAAGTCA-TTTGGCAGTTCA | 0.8 kb |
| | 141 | 01393-L25414 | Exon 11 | 1411-1412 | ATTTGACAGCCC-ATCAGGGCCGGG | 29.9 kb |
| | 171 Ж | 18997-SP0734-L25740 | Exon 12 | 1603-1604; 1639-1640 | TCCAGAATCAGA-36 nt spanning oligo-AAGAAAGAACAA | 18.6 kb |
| | 211 | 19001-L24805 | Exon 13 | 1839-1838 reverse | AATAATCTGACC-TTAAGTTGTTCT | 21.9 kb |
| | 247 | 19338-L25591 | Exon 14 | 7 nt before exon 14 | TTGATTGTCTCT-TCTCCAGGTATT | 0.3 kb |
| | 283 | 19141-L25730 | Exon 15 | 2003-2004 | TCACACAACTGG-CTTTAAAGATCA | 7.7 kb |
| | 319 | 02060-L25426 | Exon 16 | 2088-2089 | AAGCAATCCATG-GGCAAACTGTAT | 20.6 kb |
| | 358 | 01952-L25429 | Exon 17 | 2321-2322 | GGAACAGATCCT-GGTAAAGCATGC | 27.2 kb |
| | 388 | 01891-L25433 | Exon 18 | 2462-2463 | AGAAGCTGTGTT-GCAGAGTCCTGA | 16.2 kb |
| | 427 | 19005-L24809 | Exon 19 | 9 nt before exon 19 | ATCTTTGCTCTC-ATGCTGCAGGCC | 10.4 kb |
| | 466 | 01430-L25438 | Exon 20 | 2679-2680 | CGGTGGATCGAA-TTCTGCCAGTTG | 6.4 kb |
| 157 | | 01355-L25386 | Exon 21 | 2945-2946 | GAAAGGACAAGG-ACCCATGTTCCT | 12.8 kb |
| 193 | | 18998-L24802 | Exon 22 | 3159-3160 | GACTATGAAATC-ATGGAGCAGAGA | 3.7 kb |
| 226 | | 19138-L25106 | Exon 23 | 3393-3394 | AAACTCCGAAAA-ATTCAGGTAATT | 3.9 kb |
| 262 Δ | | 01958-L25397 | Exon 24 | 3470-3471 | ATGGCCTGCCCT-TGGGGATTCAGA | 1.1 kb |
| 303 | | 01371-L25399 | Exon 25 | 3583-3584 | GGCAGAAGATAA-AGAATGAAGCAG | 8.8 kb |
| 332 | | 01375-L25402 | Exon 26 | 3724-3725 | CTGTAAGCCTCC-AGAAAGATCTAT | 6.2 kb |
| 373 | | 01379-L25406 | Exon 27 | 3900-3901 | ACTGAGTCTGTA-AATAGTGTCATA | 7.3 kb |
| 405 | | 01716-L25410 | Exon 28 | 4049-4050 | TTGGCATGAGTT-ATTGTCATACTT | 3.0 kb |
| 445 | | 01387-L25413 | Exon 29 | 4227-4228 | GCACAGACCCTA-ACAGATGGCGGA | 26.5 kb® |
| 483 | | 19008-L24812 | Exon 30 | 4430-4431 | AGCTTATATTGC-AGACAAGGTGGA | 21.7 kb |
| | 157 | 01395-L25416 | Exon 31 | 4548-4549 | AAGGAGGCTGCC-CAAAGAGTCCTG | 0.6 kb |
| | 193 | 18999-L24803 | Exon 32 | 4735-4736 | AAGTAGTACAGT-CACAGCTAAATC | 3.2 kb |

| Length (nt) P034 / P035 | | SALSA MLPA probe | *DMD* exon[a] | Ligation site NM_004006.3 | **Partial sequence**[b] (24 nt adjacent to ligation site) | Distance to next probe |
|---|---|---|---|---|---|---|
| | 226 | 19140-L25108 | Exon 33 | 4882-4883 | TAACAGCTTTGA-AATTGCATTATA | 5.7 kb |
| | 263 | 01901-L25422 | Exon 34 | 4976-4977 | GCGAAAGGAAAT-GAATGTCTTGAC | 15.5 kb |
| | 303 | 15720-L25424 | Exon 35 | 5127-5128 | CACCTGAAGAGT-ATCACAGAGGTA | 0.5 kb |
| | 332 | 01416-L25427 | Exon 36 | 5317-5318 | ACATCACAAAGT-GGATCATTCAGG | 1.8 kb |
| | 372 | 03038-L26168 | Exon 37 | 5527-5526 reverse | AATGGCTGCAAA-TCGATGGTTGAG | 14.4 kb |
| | 407 | 19372-L25772 | Exon 38 | 5642-5643 | GGCTGAAATTCA-GCAGGGGGTGAA | 2.4 kb |
| | 445 | 19007-L24811 | Exon 39 | 4 nt before exon 39 | TGTTGTTTTTGA-TCAGAATGAAGA | 2.8 kb |
| | 481 Δ | 19009-L24813 | Exon 40 | 23 nt before exon 40 | TATTGATATTTT-AATAATGTCTGC | 1.1 kb |
| 149 | | 18831-L25385 | Exon 41 | 6048-6049 | GAGGGCTTGTCT-GAGGATGGGGCC | 32.0 kb |
| 187 | | 01711-L25389 | Exon 42 | 6188-6189 | AACGATGATGGT-GATGACTGAAGA | 22.6 kb |
| 220 | | 18221-L25392 | Exon 43 | 6432-6433 | GCATTGCAAAGT-GCAACGCCTGTG | 70.6 kb |
| 254 | | 01366-L25396 | Exon 44 | 6618-6619 | GAACAGTTTCTC-AGAAAGACACAA | 248.6 kb |
| 291 | | 01370-L01287 | Exon 45 | 6774-6775 | ACAGATGCCAGT-ATTCTACAGGAA | 36.3 kb |
| 325 | | 01374-L25401 | Exon 46 | 6942-6943 | AACATTGCTAGT-ATCCCACTTGAA | 2.4 kb |
| 364 | | 01378-L25405 | Exon 47 | 7046-7047 | TCTCAAACAATT-AAATGAAACTGG | 54.4 kb |
| 398 ± | | 01382-L25408 | Exon 48 | 7254-7255 | CAGTTAAATCAT-CTGCTGCTGTGG | 38.4 kb |
| 436 | | 19006-L24810 | Exon 49 | 10 nt before exon 49 | CTATATGGGTTC-TTTTCCCCAGGA | 16.9 kb |
| 476 | | 19340-L25774 | Exon 50 | 13 nt after exon 50 reverse | AGAGAATGGGAT-CCAGTATACTTA | 45.9 kb |
| | 148 | 01394-L25415 | Exon 51 | 7659-7660 | GCTCTGGCAGAT-TTCAACCGGGCT | 44.4 kb |
| | 187 | 02059-L25417 | Exon 52 | 7866-7865 reverse | CTAGCCTCTTGA-TTGCTGGTCTTG | 50.2 kb |
| | 219 | 01892-L01050 | Exon 53 | 8023-8024 | CTGAGCAGGTCT-TAGGACAGGCCA | 21.3 kb |
| | 254 | 01894-L25421 | Exon 54 | 8145-8146 | TGGCAGACAAAT-GTAGATGTGGCA | 30.3 kb |
| | 291 | 01411-L01058 | Exon 55 | 8305-8306 | AAACTCATAGAT-TACTGCAACAGT | 120.6 kb |
| | 325 Ж | 19003-SP0735-L24807 | Exon 56 | 8620-8621; 27 nt after exon 56 | AAAAGTCTCTCA-34 nt spanning oligo-CACAAATGAATT | 10.4 kb |
| | 364 | 01419-L25430 | Exon 57 | 8715-8716 | CTGAAAGATGAT-GAATTAAGCCGG | 17.8 kb |
| | 396 ± | 01423-L01070 | Exon 58 | 8846-8847 | TGAGACTGTACG-AATATTTCTGAC | 0.8 kb |
| | 436 | 01427-L25436 | Exon 59 | 9034-9035 | TAGATGAGACCC-TTGAAAGACTCC | 33.6 kb |
| | 472 | 01431-L25439 | Exon 60 | 9204-9205 | GCGCCTCTGAAA-GAGAACGTGAGC | 96.0 kb |
| 165 | | 01356-L25387 | Exon 61 | 9354-9355 | AGGCAGCTGCAT-GAAGCCCACAGG | 25.0 kb |
| 199 | | 01897-L25390 | Exon 62 | 9423-9424 | GGTCCCTGGGAG-AGAGCCATCTCG | 62.6 kb |
| 241 | | 01364-L25393 | Exon 63 | 9482-9483 | TCAAACAACTTG-CTGGGACCATCC | 37.9 kb |
| 268 | | 01368-L25398 | Exon 64 | 9576-9575 reverse | TTCTGCAGTCTT-CGGAGTTTCATG | 13.6 kb |
| 312 | | 19002-L26038 | Exon 65 | 9774-9775 | GATATGTGTCTG-AACTGGCTGCTG | 2.9 kb |
| 341 | | 01376-L25403 | Exon 66 | 9835-9836 | TCCTGTCTTTTA-AAACTGGCATCA | 2.6 kb |
| 381 | | 01960-L25407 | Exon 67 | 10016-10015 reverse | GGACACTTGGCT-CAATGTTACTGC | 21.1 kb |
| 413 | | 02482-L28035 | Exon 68 | 10093-10094 | TAGACTGGATGA-GACTGGAACCCC | 2.4 kb |
| 454 | | 19341-L25594 | Exon 69 | 23 nt before exon 69 | GAAATACATACG-TGTTTGTTTTTG | 1.8 kb |
| 493 | | 01392-L27986 | Exon 70 | 10414-10415 | ATCCCGAATGG-GCTACCTGCCAG | 0.7 kb |
| | 166 Ж | 18996-SP0733-L25739 | Exon 71 | 38 nt before exon 71; 8 nt before exon 71 | CGGCTGAGTTTG-30 nt spanning oligo-TTTTGCAGTCCC | 4.4 kb |
| | 199 | 01949-L25418 | Exon 72 | 10522-10523 | CCCCTCAGCTTT-CACACGATGATA | 1.2 kb |
| | 239 | 01893-L25419 | Exon 73 | 10600-10599 reverse | GCTATCATTTAG-ATAAGATCCATT | 2.9 kb |
| | 269 | 01902-L25423 | Exon 74 | 10722-10723 | GCCCAGATCTTG-ATTTCCTTAGAG | 22.1 kb |

| Length (nt) P034 / P035 | SALSA MLPA probe | *DMD* exon[a] | Ligation site NM_004006.3 | Partial sequence[b] (24 nt adjacent to ligation site) | Distance to next probe |
|---|---|---|---|---|---|
| 310 | 01413-L25425 | Exon 75 | 10914-10915 | GCTGAGCTCATT-GCTGAGGCCAAG | 1.0 kb |
| 342 | 01417-L25428 | Exon 76 | 11092-11093 | CTACCTCTCTAC-AGAGGTCCGACA | 12.2 kb |
| 381 | 01421-L25432 | Exon 77 | 11193-11194 | CCCCAGGACACA-AGCACAGGGTTA | 7.6 kb |
| 413 | 19342-L25595 | Exon 78 | 43 nt after exon 78 | TCTGTCTGTCTC-ATCCTGCTTTTT | 6.2 kb |
| 453 | 19142-L25437 | Exon 79 | 12818-12819 | CCATCCTTTGCA-TTTCTCTGCGAG | |
| | *stop codon 11293-11295 (Exon 79)* | | | | |

**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.

Δ More variable. This probe is sensitive to experimental conditions. Aberrant results should be treated with caution.
± SNP rs398124074 could influence the 396 nt probe signal (P035). SNP rs756370588 could influence the 391 nt probe signal and SNP rs398124041 could influence the 398 nt probe signal (P034). In case of apparent deletions, it is recommended to sequence the regions targeted by these probes.
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. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

## Related SALSA MLPA Probemixes

P048 LMNA/MYOT/ZMPSTE24    Laminopathies; contains probes for the *LMNA*, *MYOT*, *ZMPSTE24* and *CAV3* genes.

P116 SGC    Limb girdle muscular dystrophy (LGMD); contains probes for the *SGCA*, *SGCB*, *SGCD*, *SGCG* and *FKRP* genes.

P176 CAPN3    Limb girdle muscular dystrophy (LGMD); contains probes for the *CAPN3* gene.

P268 DYSF    Limb girdle muscular dystrophy (LGMD); contains probes for the *DYSF* gene.

P436 ANO5    Limb girdle muscular dystrophy (LGMD); contains probes for the *ANO5* gene.

## References

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## Selected publications using SALSA MLPA Probemix P034 DMD-1 and P035 DMD-2

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- Gatta V et al. (2005). Identification of deletions and duplications of the DMD gene in affected males and carrier females by multiple ligation probe amplification (MLPA). *Hum Genet*. 117:92-98.
- Han S et al. (2020). Population-Wide Duchenne Muscular Dystrophy Carrier Detection by CK and Molecular Testing. *Biomed Res Int*. 2020.
- Lee SH et al. (2015). Clinical and genetic characterization of female dystrophinopathy. *J Clin Neurol*. 11:248-251.
- Luce LN et al. (2016). MLPA analysis of an Argentine cohort of patients with dystrophinopathy: Association of intron breakpoints hot spots with STR abundance in DMD gene. *J Neurol Sci*. 365:22-30.
- Nakamura A et al. (2016). Deletion of exons 3-9 encompassing a mutational hot spot in the DMD gene presents an asymptomatic phenotype, indicating a target region for multiexon skipping therapy. *J Hum Genet*. 61:663-667.
- Selvatici R et al. (2021). Ethnicity-related DMD Genotype Landscapes in European and Non-European Countries. *Neurol Genet*. 7:e536.
- Traverso M et al. (2018). Clinical and molecular consequences of exon 78 deletion in DMD gene. *J Hum Genet*. 63:761-4.
- Zhang J et al. (2019). Genetic analysis of 62 Chinese families with Duchenne muscular dystrophy and strategies of prenatal diagnosis in a single center. *BMC medical genetics*. 20:180.


| P034 product history | |
|---|---|
| *Version* | *Modification* |
| B2 | Two probes have a small change in length; no change in sequence detected. |
| B1 | All reference probes and the probes for *DMD* exons 8, 22, 23, 30, 49, 50, 65 and 69 have been replaced. |
| A3 | The 88 and 96 nt control fragments have been replaced (QDX2). |
| A2 | Four control fragments at 88-96-100-105 nt have been added. |
| A1 | First release. |


| P035 product history | |
|---|---|
| *Version* | *Modification* |
| B1 | All reference probes and the probes for *DMD* exons 12-15, 19, 32, 33, 39, 40, 56, 71, 78, and 79 have been replaced. |
| A3 | The 88 and 96 nt control fragments have been replaced (QDX2). |
| A2 | Four control fragments at 88-96-100-105 nt have been added. |
| A1 | First release. |


| Implemented changes in the product description |
|---|
| Version B2/B1-08 –  19 January 2023 (04P)<br> - Section Note: Slope correction in samples with large deletions updated.<br> - Section of P034/P035 specific notes updated.<br> - Warning added to Table 1 and Table 2 for more variable probes, 142 nt probe 01353-L25384, and 481 nt probe 19009-L24813.<br> - Various minor textual changes.<br><br>Version B2/B1-07 – 16 September 2021 (04P) |

- Section of P034/P035 specific notes updated.

Version B2/B1-06 – 24 March 2021 (04P)
- Product description rewritten and adapted to a new template.
- Intended purpose updated.
- Clinical background and Performance characteristics sections updated.
- Reference samples section updated.
- Note for slope correction added to the data analysis section.
- Section of selected publications updated.
- UK added to the list of countries in Europe that accept the CE mark.

Version B2/B1-05 – 21 September 2020 (02P)
- Warning added to Table 1a and Table 2 for SNPs that could influence probe signal of 391 nt probe 19004-L24808, and 398 nt probe 01382-L25408.

Version B2/B1-04 – 13 July 2020 (02P)
- Product description rewritten and adapted to a new template.
- Information about transcript variants updated to new versions of the NM_ reference sequences.
- Ligation sites of the probes targeting the *DMD* gene updated according to new versions of the NM_ reference sequences.
- Costa Rica added as country with IVD status.
- Related SALSA MLPA probemixes section updated.

Version B2/B1-03 – 24 October 2018 (04)
- Product description restructured and adapted to a new template.
- Warning added to Table 1a and Table 2 for more variable probe, 262 nt probe 01958-L25397.
- Warning added to Table 1b and Table 2 for SNP that could influence probe signal, 396 nt probe 01423-L01070.
- Warning removed from Table 1b and Table 2, for variable probe, 239 nt probe 01893-L25419.
- Several references were removed, 7 references were added.
- Section on Artificial positive control DNA sample was replaced by section on Positive control DNA samples.
- Minor textual changes.

| More information: www.mrcholland.com; www.mrcholland.eu | |
|---|---|
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| IVD | EUROPE* CE<br>COLOMBIA<br>COSTA RICA<br>ISRAEL<br>MOROCCO |
|---|---|
| RUO | ALL OTHER COUNTRIES |

*comprising EU (candidate) member states and members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.