Product Description

SALSA® MS-MLPA® Probemix ME029-C1 FMR1-AFF2

To be used with the MS-MLPA General Protocol with changes as indicated below.

Version C1

As compared to version B3, target probes for *FMR1* and *AFF2* have been added. The digestion control probes and the probes detecting autosomal chromosomal locations have been replaced. Multiple reference probes have been replaced. For complete product history see page 11.

Catalogue numbers:

- **ME029-025R:** SALSA MS-MLPA Probemix ME029 FMR1-AFF2, 25 reactions.
- **ME029-050R:** SALSA MS-MLPA Probemix ME029 FMR1-AFF2, 50 reactions.
- **ME029-100R:** SALSA MS-MLPA Probemix ME029 FMR1-AFF2, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit, SALSA HhaI (SMR50), SALSA Low pH MLPA buffer, 60 mM NaOH (not provided) and Coffalyser.Net data analysis software. Please note: the SALSA MLPA buffer (yellow cap) in the SALSA MLPA reagent kit is not used. 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 MS-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.

Please note that 60 mM NaOH (not provided) and Low pH MLPA buffer (provided separately) should be used for this ME029 MLPA kit instead of the SALSA MLPA buffer provided with the reagent kit.
Complete denaturation of very long trinucleotide repeats requires the use of 60 mM NaOH in combination with Low pH MLPA buffer. The standard MS-MLPA can be followed, but use the following instructions for the DNA Denaturation step (section 6.2. in MS-MLPA protocol) and Hybridisation reaction step (section 6.3 in MS-MLPA protocol):

6.2. DNA DENATURATION (DAY 1):

- Prepare 60 mM NaOH solution. 60 mM NaOH solution is not provided and should be prepared fresh for each experiment. 60 mM NaOH solutions can also be aliquoted and frozen for single use. We recommend a minimum aliquot size of 0.5 ml to prevent pH changes due to CO2 uptake from air. 1 M NaOH solutions can be bought from several providers, e.g. Sigma S2770.
- Label 0.2 ml tubes, strips or plates.
- Prepare each sample by mixing **2 µl of 60 mM NaOH solution** (not provided) and 3 µl DNA sample containing 50-250 ng of sample DNA. If necessary, use TE to reach the desired volume. Add 5 µl to each tube. Use TE for a No DNA control.
- Place tubes in thermocycler; start MS-MLPA thermocycler program steps 1-2 (see section 6.1).
- Ensure samples are at 25°C before removing tubes from the thermocycler.

6.3. HYBRIDISATION REACTION (DAY 1):

- Prepare hybridisation master mix. For each reaction, mix: 1.5 µl Low pH MLPA buffer (purple cap) + 1.5 µl probemix (black cap). Mix well by pipetting or vortexing.
- After DNA denaturation, add 3 µl hybridisation master mix to each reaction. Accurate pipetting is critical. Mix well by pipetting gently up and down.
- Continue thermocycler program with steps 3-4.

Follow the standard MS-MLPA protocol from 6.4. **LIGATION & LIGATION-DIGESTION REACTIONS (DAY 2)** onwards (see p.12 for short protocol).

General information
The SALSA MS-MLPA Probemix ME029 FMR1-AFF2 is a **research use only (RUO)** assay for the detection of aberrant methylation of one or more sequences of the *FMR1* and *AFF2* genes. This probemix can also be used to detect deletions/duplications in the aforementioned genes.

The *FMR1 (FRAXA)* gene (17 exons) spans ~39 kb on Xq27.3. The *AFF2 (FMR2 , FRAXE)* gene (21 exons) spans ~500 kb of genomic DNA and is located in Xq28, at a distance of ~600 kb telomeric of *FMR1*. The most common defect in these two genes is an expansion of a trinucleotide repeat sequence in exon 1.

For the *FMR1* gene, the trinucleotide repeat motif is CGG, where alleles are classified as normal (unmethylated alleles; 5-44 repeats), intermediate (45-54 repeats), premutation (55-200 repeats) and full-mutation (>200 repeats) (Jorge et al. 2013). Hyper-expansion of *FMR1* CGG repeats to >200 is accompanied by hypermethylation of the promoter and gene silencing, which results in Fragile X syndrome (FXS) due to a reduced or absent *FMR1* protein (FMRP) (Liu et al. 2021). FXS has an X-linked dominant inheritance pattern and is the most common inherited form of intellectual disability and autism spectrum disorders (Pugin et al. 2017) with a population prevalence of about 1/4000–9000 males and 1/7000–15,000 females (Rzonka et al. 2016). Individuals with a repeat range of 55 to 200 repeats are said to be carriers of a premutation, which does not affect the promoter methylation. However, it can lead to fragile X-associated tremor/ataxia syndrome (FXTAS) affecting primarily men which is characterised by late-onset (>50 years), progressive cerebellar ataxia and intention tremor followed by cognitive impairment. Premutations can also lead to fragile X-associated primary ovarian insufficiency (FXPOI) in females, which manifests as cessation of menstrual periods before reaching the age of 40 (Pugin et al. 2017).

In the *AFF2* gene a CCG expansion can occur. In the normal population, the number of repeats varies between 6 and 35 and is increased to >200 in the Fragile XE Syndrome (FRAXE). To what extent the alleles with CCG repeats in the range between 36 and 199 may exhibit a pathogenic role remains elusive. FRAXE is the most

prevalent form of non-syndromic X-linked intellectual disability with a milder to borderline phenotype as compared to FXS. It has also been described that microdeletions in *AFF2* may be a significant cause of premature ovarian failure (Murray et al. 1999). However, in contrast to FXS, reports of *AFF2* full expansions are very rare and the dynamics of this repeat is not as clearly characterized as that of *FMR1* leading to poorly defined phenotypes (Jorge et al. 2013).

Results obtained with this SALSA MS-MLPA Probemix ME029 FMR1-AFF2 have shown that *FMR1* promoter methylation can be easily detected in DNA samples from males with a *FMR1* full mutation (>200 repeats). However, a distinction between premutation alleles and normal alleles cannot be made. For female DNA samples, the presence of an imprinted X-chromosome complicates analysis and can easily result in false positive results. In our studies, the variation in results was too high to reliably detect all full mutation female samples. For methylation quantification, this product can be used for the analysis of the methylation status of the promoters of *FMR1* and *AFF2* in male samples only. Thus, even if it is not possible to measure the length of the repeat by MLPA, the methylation at certain CpG sequences in the promoter region of these genes can still be quantified by MS-MLPA.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1384/ (*FMR1*-Related Disorders)

This SALSA MS-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
Matched Annotation from NCBI and EMBL-EBI (MANE): http://www.ncbi.nlm.nih.gov/refseq/MANE/
Tark – Transcript Archive (MANE) database: http://tark.ensembl.org/
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Exon numbering
The *FMR1* and *AFF2* exon numbering used in this ME029-C1 FMR1-AFF2 product description is the exon numbering derived from MANE project (release version 1.0) based on Mane Select transcripts NM_002024.6 and NM_002025.4 respectively. 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 MS-MLPA Probemix ME029-C1 FMR1-AFF2 contains 48 (MS-)MLPA probes with amplification products between 130 and 472 nucleotides (nt). 20 probes are specific for the *FMR1* gene and 14 for the *AFF2* gene. Nine of these probes contain an HhaI recognition site and provide information on the methylation status of the exon 1 region of *FMR1* (six probes) and *AFF2* (three probes). All probes present will also give information on copy number changes in the analysed sample. In addition, ten reference probes are included that are not affected by HhaI digestion and detect genes located outside the Xq27.3 and Xq28 regions. Also, 2 digestion control probes are included in this probemix indicating whether or not restriction endonuclease digestion in the MS-MLPA reaction was complete. Furthermore, two probes with autosomal targets are included for the *TGFB1I1* gene (16p11) and one for the *PCCB* gene (3p22). Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

This probemix contains ten quality control fragments generating amplification products between 64 and 118 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and two chromosome Y-specific fragments (see table below). More information on how to interpret observations on these control fragments can be found in the MS-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)
105-118	Y-fragments (Y chromosome specific)

Warning: DNA denaturation control probes

Each MLPA probemix of MRC Holland contains two probes at 88 and 96 nt that provide a warning in case the sample DNA is not completely denatured, e.g. as a result of the presence of 40 mM salt or more in the sample DNA. These control probes detect a sequence that is located in the strongest CpG islands of the human genome. However, patients with a full mutation allele of *FMR1* or *AFF2* have an unusual sequence of more than 500 nt with 100% CG residues which is extremely difficult to denature. Our 88 and 96 nt control fragments fail to warn for situations in which this expanded allele is not completely denatured and such situations can lead to results in which copy number analysis shows an apparent deletion of exon 1.

When low copy number ratios for exon 1 in male samples are reported by the Coffalyser.Net software and the other *FMR1* exons show normal copy number ratios, this will be an indication of incomplete denaturation, possibly due to a high repeat number. In order to prevent such incomplete DNA denaturation in full mutation patients, the use of 60 mM NaOH (not provided) in combination with the Low pH MLPA buffer provided with the ME029 kit is essential. However, in increased salt conditions the use of 60 mM NaOH in combination with the provided Low pH MLPA buffer may not be able to prevent this incomplete DNA denaturation. Therefore, in male samples with lower copy number ratios than expected (ratios <0.80), caution is warranted in interpretation of the results.

MS-MLPA technique

The principles of the MS-MLPA technique (Nygren et al. 2005, Schouten et al. 2002) are described in the MS-MLPA General Protocol (www.mrcholland.com).

MS-MLPA technique validation

Internal validation of the MS-MLPA technique using 16 DNA samples from healthy individuals of the same sex is required, in particular when using MS-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.

Results of MS-MLPA are highly dependent on the HhaI enzyme used. HhaI enzymes that are resistant to heat inactivation are NOT compatible with the MS-MLPA technique and will give aberrant results. These include, but may not be limited to, Thermo Fisher Scientific enzymes HhaI, ANZA 59 HhaI, and FastDigest HhaI. We recommend using SALSA HhaI enzyme (SMR50) as this restriction enzyme has been validated for use with MS-MLPA by MRC Holland.

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 MS-MLPA General Protocol.

Reference samples

A sufficient number (≥3) of reference samples should be included in each MS-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. When selecting reference samples, please note that methylation patterns may vary between tissue types and even age groups! Reference samples should be derived from different unrelated individuals who are from families without a history of Fragile X syndrome or Fragile XE syndrome. It is recommended to use samples

of the same sex to facilitate interpretation. More information regarding the selection and use of reference samples can be found in the MS-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 NA09145, NA09237 and NA20231 from the Coriell Institute have been tested with this ME029-C1 probemix at MRC Holland and can be used as a positive control sample to detect *FMR1* full mutations (NA09145 and NA09237). However, a premutation sample (NA20231) shows no difference with a healthy sample.

The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Chromosomal position of copy number alteration	Altered target genes in ME029-C1	Expected alteration
NA09145	Coriell Institute	Xq27.3	*FMR1*	Full-mutation (hypermethylation of FMR1 methylation specific probes)
NA09237	Coriell Institute	Xq27.3	*FMR1*	Full-mutation (hypermethylation of FMR1 methylation specific probes)
NA20231	Coriell Institute	Xq27.3	none	Premutation (no methylation of FMR1 methylation specific probes, no difference with normal sample)

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 copy number 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 when **reference samples of the same sex** have been used:

Copy number status		Final ratio (FR)
Autosomal sequences and X chromosome sequences in females	**X chromosome sequences in males**	
Normal	Normal	0.80 < FR < 1.20
Homozygous deletion	Deletion	FR = 0
Heterozygous deletion	n.a.	0.40 < FR < 0.65
Heterozygous duplication	n.a.	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	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.

Note: Four autosomal probes are included in this probemix (including two digestion control probes). These can be used for detection of copy number changes of the entire X-chromosome.

- 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. Please refer to the warning in page 2.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 MS-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.
- Digestion Control Probes. The target sequences of the digestion control probes are unmethylated in most blood-derived DNA samples. The signals of the digestion control probes should be gone upon complete digestion by HhaI.
- mRNA levels. We have no data showing that methylation detected by a particular probe indeed influences the corresponding mRNA levels.
- 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.

NOTE: In case digestion control probes are not fully digested (>0.05), please contact info@mrcholland.com for more information.

ME029 specific note:

Complete denaturation of very long *FMR1* or *AFF2* trinucleotide repeats requires the use of 60 mM NaOH (not provided) in combination with the Low pH MLPA buffer provided with the ME029 kit is essential.
Limitations of the procedure

- MS-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 MS-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.
- An MS-MLPA probe targets a single specific HhaI site in a CpG island; if methylation is absent for a particular CpG-site, this does not necessarily mean that the whole CpG island is unmethylated!
- Rare cases are known in which apparent methylation as detected by an MS-MLPA probe proved to be due to a sequence change in or very near the HhaI site.
- This product can be used for the analysis of the methylation status of the promoters of *FMR1* and *AFF2* in male samples only.
- A distinction between *FMR1* premutation alleles and normal alleles cannot be made.

Confirmation of results

Confirmation of methylation status can be performed with another technique, such as MSP (methylation-specific PCR), pyrosequencing, digestion-based PCR assays, etc. 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 MS-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.

***FMR1 and AFF2* mutation database**

We strongly encourage users to deposit positive results in the https://databases.lovd.nl/shared/genes/FMR1 & https://databases.lovd.nl/shared/genes/AFF2 databases. 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 *AFF2* exons 3 and 5 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MS-MLPA Probemix ME029-C1 FMR1-AFF2

Length (nt)	SALSA MLPA probe	HhaI site	% methylated in normal blood-derived male DNA	% expected signal reduction	Chromosomal position (hg18)[a]		
					Other	*FMR1*	*AFF2*
64-118	Control fragments – see table in probemix content section for more information						
130 *	Reference probe 22528-L31699				Xq		
136 * «	**FMR1 probe** 22610-L31824					**Exon 1**	
142 «	**FMR1 probe** 12915-L14360	+	0%	100%		**Exon 1**	
148 * «	**AFF2 probe** 22724-L32005						**Exon 1**
155 ¥ «	**FMR1 probe** 22786-L12030	+	0%	100%		**Exon 1**	
160 «	**FMR1 probe** 03722-L03182	+	0%	100%		**Exon 1**	
166 *	Reference probe 04423-L05579				Xp		
171 ¥ « ‡	**AFF2 probe** 22601-L32328	+	0%	100%			**Exon 1**
178 * π	Digestion control probe 22371-L21055	+	0%	100%	**8p21**		
184 ¥ «	**FMR1 probe** 12916-L32329	+	0%	100%		**Exon 1**	
190 * «	**FMR1 probe** 04037-L32286					**Exon 1**	
196 *	Reference probe 06111-L05566				Xq		
203 *	**TGFB1I1 probe** 13121-L31675				**16p11**		
214 «	**FMR1 probe** 03725-L13188	+	0%	100%		**Exon 1**	
220	**AFF2 probe** 04029-L14362						**Exon 20**
226 «	**FMR1 probe** 03728-L14363					**Exon 3**	
232 *	Reference probe 21142-L29424				Xp		
238 * «	**FMR1 probe** 22612-L31825					**Exon 1**	
245 «	**AFF2 probe** 03736-L03196	+	0%	100%			**Exon 1**
253 * «	**FMR1 probe** 22613-L31827					**Exon 1**	
259 «	**AFF2 probe** 03733-L14365	+	0%	100%			**Exon 1**
267 * «	**FMR1 probe** 22631-L32330					**Exon 2**	
276 *	Reference probe 02900-L26167				Xq		
283	**AFF2 probe** 00493-L00066						**Exon 3**
290 * «	**FMR1 probe** 22632-L32331					**Exon 4**	
295 *	Reference probe 19391-L32337				Xp		
301	**AFF2 probe** 03739-L03199						**Exon 4**
310 *	**FMR1 probe** 22633-L31840					**Exon 13**	
317 *	Reference probe 03045-L32332				Xp		
323 * «	**AFF2 probe** 22634-L31841						**Exon 1**
331 * «	**FMR1 probe** 22635-L32333					**Exon 7**	
337	**AFF2 probe** 12914-L03200						**Exon 7**
345 *	**FMR1 probe** 22636-L31843					**Exon 12**	
355	**AFF2 probe** 03741-L03201						**Exon 8**
365 *	**AFF2 probe** 22637-L31844						**Exon 2**
372 * π	Digestion control probe 21589-L32334	+	0%	100%	**2q12**		
378 *	Reference probe 01282-L32475				Xq		
384 ¥	**FMR1 probe** 13214-L32336					**Exon 15**	
391 *	**AFF2 probe** 22638-L31845						**Exon 5**
398 ¥	**FMR1 probe** 12913-L03721					**Exon 9**	
409	**AFF2 probe** 03742-L14538						**Exon 10**
418 *	Reference probe 06473-L05999				Xp		
427 *	**FMR1 probe** 22639-L31846					**Exon 14**	
436	**AFF2 probe** 03743-L03203						**Exon 14**
445 *	**FMR1 probe** 22640-L31847					**Exon 17**	
456 *	**FMR1 probe** 22641-L31848					**Exon 10**	
465 *	**PCCB probe** 21408-L29885				**3q22**		
472	Reference probe 02915-L02309				Xq		

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

* New in version C1.
¥ Changed in version C1. Minor alteration, no change in sequence detected.
« 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.
π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
‡ This probe contains more than one GCGC motif for HhaI. Loss of probe peak signal (indicative of absence of methylation) will occur not only if all sites are unmethylated but also if one of the sites is unmethylated and the other methylated.

Table 2. ME029-C1 target probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene/Exon[a]	HhaI site	Ligation site	Distance to next probe
		FMR1		**NM_002024.6**	
253 «	22613-L31827	Exon 1		302 nt before exon 1	0.3 kb
136 «	22610-L31824	Exon 1		42 nt before exon 1	0.1 kb
214 «	03725-L13188	Exon 1	+	8-9	0.2 kb
238 «	22612-L31825	Exon 1		254-255	0.1 kb
		start codon		*262-264 (exon 1)*	
160 «	03722-L03182	Exon 1	+	304-305	0.1 kb
184 «	12916-L32329	Exon 1	+	113 nt after exon 1 reverse	0.1 kb
142 «	12915-L14360	Exon 1	+	190 nt after exon 1	0.1 kb
190 «	04037-L32286	Exon 1		331 nt after exon 1	0.1 kb
155 «	22786-L12030	Exon 1	+	466 nt after exon 1	9.2 kb
267 «	22631-L32330	Exon 2		313-314	3.6 kb
226 «	03728-L14363	Exon 3		385-386	2.8 kb
290 «	22632-L32331	Exon 4		495-494 reverse	1.8 kb
331 «	22635-L32333	Exon 7		804-805	2.6 kb
398	12913-L03721	Exon 9		1091-1092	3.9 kb
456	22641-L31848	Exon 10		1220-1219 reverse	1.5 kb
345	22636-L31843	Exon 12		1419-1420	2.5 kb
310	22633-L31840	Exon 13		1479-1480	2.6 kb
427	22639-L31846	Exon 14		1640-1641	1.7 kb
384	13214-L32336	Exon 15		1797-1798	3.8 kb
445	22640-L31847	Exon 17		2039-2040	551.8 kb
		stop codon		*2158-2160 (exon 17)*	
		AFF2		**NM_002025.4**	
148 «	22724-L32005	Exon 1		87 nt before exon 1 reverse	0.4 kb
259 «	03733-L14365	Exon 1	+	321-322	0.4 kb
		start codon		*482-484 (exon 1)*	
245 «	03736-L03196	Exon 1	+	226 nt after exon 1	0.1 kb
323 «	22634-L31841	Exon 1		305 nt after exon 1	0.2 kb
171 « ‡	22601-L32328	Exon 1	+	501 nt after exon 1	150.4 kb
365	22637-L31844	Exon 2		626-627	10.1 kb
283	00493-L00066	Exon 3		980-981	147.7 kb
301	03739-L03199	Exon 4		1547-1546 reverse	27.8 kb
391	22638-L31845	Exon 5		1633-1634	5.7 kb
337	12914-L03200	Exon 7		1729-1728 reverse	42.5 kb
355	03741-L03201	Exon 8		1783-1784	67.8 kb
409	03742-L14538	Exon 10		2006-2005 reverse	13.1 kb
436	03743-L03203	Exon 14		3452-3453	20.6 kb
220	04029-L14362	Exon 20		4130-4131	
		stop codon		*4415-4417 (exon 21)*	

[a] See section Exon numbering on page 3 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.
‡ This probe contains more than one GCGC motif for HhaI. Loss of probe peak signal (indicative of absence of methylation) will occur not only if all sites are unmethylated but also if one of the sites is unmethylated and the other methylated.

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 3. Sequences detected by the ME029-C1 FMR1-AFF2 probes

Length (nt)	SALSA MS-MLPA probe	Sequence with HhaI site
214	03725-L13188	CCTTCCGGGGGTTCGGCCTCAGTCA-GGCGCTCAGCTCCGTTTCGGTTTCACTTCCGGTGGAG
160	03722-L03182	GGTGCGGGGCTCCAATGGCGCTT-TCTACAAGGTACTTGGCTCTAGGGCAGGCCCCATCTTC
184	12916-L32329	CGCTTCCCTCCCAACAACATCCCGCCGAGCGTGCCCT-GGCACCCAGGCGCGGTGCTCGGGAAGAGG
142	12915-L14360	CTGTTGGAAGCCCCTCTCCGACTCCGA-GAGGCCCTAGCGCCTATCGAAATGAGAGACCAGCGAG
155	22786-L12030	CCTCCTGCAGCGCCAAGAGGGCTTCA-GGTCTCCTTTGGCTTCTCTTTTCCGGTCTAGCATTGGGACT
259	03733-L14365	GGAGCACAGGACCAGACACCTCCA-GCGCCCGCTGCTGCTGCCGATGCGGCCCGGACAC
245	03736-L03196	CGGACCCGAAAGCGTCGCTTT-GCGCTCCAAGTCTAAAGAGTTGCATTTGGCTCACATCGAA
171 ‡	22601-L32328	GTACCCGCTGCCGCCACCTCCATCCCTGCGCT-GCGCGGCGCGCACACCTTTCCTTCTGCCGACTCAGCC

‡ This probe contains more than one GCGC motif for HhaI. Loss of probe peak signal (indicative of absence of methylation) will occur not only if all sites are unmethylated but also if one of the sites is unmethylated and the other methylated.

The HhaI sites are marked with grey. Ligation sites are marked with –. Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

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ME029 product history	
Version	*Modification*
C1	Target probes for *FMR1* and *AFF2* have been added. The digestion control probes and the probes detecting autosomal chromosomal locations have been replaced. Flanking probes have been removed. Multiple references probes have been replaced.
B3	Small oligo modification (208 nt probe), no change in length nor sequence detected.
B2	The 88, 96 and 118 nt control fragments have been replaced (QDX2). One probe has a small change in length.
B1	Target probes have been added and replaced to increase specificity. Multiple reference probes have been replaced.
A1	First release.

Implemented changes in the product description
Version C1-01 – 03 July 2023 (04M) - Product description rewritten and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - Product description adapted to a new ME029-protocol. - Introduction of the use of 60 mM NaOH in combination with the Low pH MLPA buffer.

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

ME029-C1 FMR1-AFF2 MS-MLPA PROTOCOL – IN BRIEF

1. DNA DENATURATION
   - Prepare 60 mM NaOH solution. 60 mM NaOH solution is not provided and should be prepared fresh for each experiment. 60 mM NaOH solutions can also be aliquoted and frozen for single use. We recommend a minimum aliquot size of 0.5 ml to prevent pH changes due to CO_2 uptake from air. 1 M NaOH solutions can be bought from several providers, e.g. Sigma S2770.
   - Mix **2 µl of 60 mM NaOH solution** (not provided) and 3 µl DNA sample containing 50-250 ng of sample DNA. If necessary, use TE to reach the desired volume.
   - Heat for 5 minutes at 98°C.
2. HYBRIDISATION OF PROBES TO SAMPLE DNA
   - Cool down to room temperature, open tubes
   - Add 3 µl hybridisation master mix*
   - Incubate 1 minute at 95°C and hybridise for 16 hours at 60°C
3. LIGATION AND LIGATION-DIGESTION OF HYBRIDISED PROBES
   - Lower thermocycler temperature to 20°C, open tubes
   - Add 13 µl of ligase buffer A master mix*
   - Mix well and transfer 10 µl to a second tube
   - Place samples in thermocycler, heat to 48°C and when this temperature is reached:
   - Add 10 µl Ligase-65 master mix* to the first tube (undigested reaction)
   - Add 10 µl Ligase-digestion master mix* to the second tube (digested reaction)
   - Incubate 30 minutes at 48°C
   - Heat inactivate the ligase and HhaI enzymes: 5 minutes 98°C
4. PCR AMPLIFICATION OF LIGATED PROBES
   - Cool down to room temperature, open tubes
   - Add 5 µl polymerase master mix* at room temperature
   - Start PCR (35 x {95°C 30 seconds, 60°C 30 seconds, 72°C 60 seconds}, 72°C 20 minutes, 15°C pause)
5. FRAGMENT SEPARATION BY CAPILLARY ELECTROPHORESIS
6. ANALYSE RESULTS WITH COFFALYSER.NET

* Master mixes:

- Hybridisation: 1.5 µl SALSA probemix +1.5 µl **Low pH MLPA buffer**, per reaction
- Ligase buffer A: 3 µl ligase buffer A + 10 µl ultrapure water, per reaction
- Ligase-65: 1.5 µl ligase buffer B + 8.25 µl ultrapure water + 0.25 µl Ligase-65, per reaction
- Ligase-digestion: 1.5 µl ligase buffer B + 7.75 µl ultrapure water + 0.25 µl Ligase-65 + 0.5 µl HhaI, per reaction
- Polymerase: 3.75 µl ultrapure water + 1 µl PCR primer mix + 0.25 µl SALSA polymerase, per reaction

For more information please see the MS-MLPA® General Protocol (www.mrcholland.com). This ME029 protocol differs from the normal MS-MLPA protocol only in step 1 and 2, the DNA Denaturation and hybridisation of probes to the sample DNA. The use of 60 mM NaOH in combination with Low pH MLPA buffer is essential for complete DNA denaturation of full mutation samples.