Product Description

SALSA® MLPA® Probemix P070-B3 Subtelomeres Mix 2B

To be used with the MLPA General Protocol.

Version B3
For complete product history see page 12.

Catalogue numbers:

- **P070-025R:** SALSA MLPA Probemix P070 Subtelomeres Mix 2B, 25 reactions.
- **P070-050R:** SALSA MLPA Probemix P070 Subtelomeres Mix 2B, 50 reactions.
- **P070-100R:** SALSA MLPA Probemix P070 Subtelomeres Mix 2B, 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 P070 Subtelomeres Mix 2B is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletion(s) or duplication(s) in subtelomeric regions in genomic DNA isolated from human peripheral whole blood specimens, buccal swabs, (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, foetal blood or products of conception free from maternal contamination. P070 Subtelomeres Mix 2B is intended to confirm a potential cause for and clinical diagnosis of intellectual disability, developmental delay, congenital abnormalities and/or pregnancy loss and for molecular genetic testing of at-risk family members.

It is recommended that results of P070 Subtelomeres Mix 2B are confirmed with P036 Subtelomeres Mix 1. Copy number variations (CNVs) detected with one or both probemixes should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method.

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.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation 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.
[2] To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

Clinical background
In the general population, the prevalence of intellectual disability and developmental delay is estimated at 1-3% and the prevalence of congenital anomalies at 2-3% (Castells-Sarret et al. 2017). Clinical signs can include dysmorphic features, language impairment, seizures, learning disabilities, behavioural disturbances and

autism spectrum disorders. It has been recognized that worldwide the genetic etiology of individuals with non-syndromic intellectual disability remains undetermined in the majority of cases. Determining the etiology of intellectual disability and developmental delay is important and useful for pediatric neurologists, geneticists, pediatricians, and patients' families because it allows assessment of recurrence risk, appropriate genetic counselling, and focus on treatment options and prognosis.

Aberrant copy numbers of subtelomeric regions, e.g. due to an unbalanced translocation, are a frequent cause of intellectual disability, developmental delay and/or congenital abnormalities lacking distinct syndromic features. Subtelomeric regions are more likely to be involved in copy number changes as these are less likely to be lethal due to the low amount of critical genes located in these regions. The differences in size and breakpoint location of chromosomal CNVs make the phenotype highly variable among patients, ranging from a non-viable foetus to a phenotypically normal individual. Chromosomal anomalies of the foetus are found in almost half of the miscarriages and are the most common reason for pregnancy loss (Bernatowicz et al. 2019). More information can be found on Decipher (http://decipher.sanger.ac.uk/) and in the references listed at the end of this product description.

Probemix content

The SALSA MLPA Probemix P070-B3 Subtelomeres Mix 2B contains 46 MLPA probes with amplification products between 132 and 490 nucleotides (nt). This includes 41 probe(s) for the subtelomeric regions, no probes are present for the subtelomeric regions on the short p-arm of the 5 acrocentric chromosomes (13, 14, 15, 21 and 22). For these, an extra probe is included detecting the q-arm, close to the centromere. The subtelomeric probes for the X and Y chromosome are identical as they detect sequences in the pseudo-autosomal regions (PAR1 and PAR2) which are identical in chromosome X and Y. More information is present in Table 1 of this document. Complete probe sequences are available online (www.mrcholland.com).

This probemix contains ten quality control fragments generating amplification products between 64 and 121 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 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)
105-121	Y-fragments (Y chromosome specific)

MLPA technique

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

MLPA technique validation

Internal validation of the MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. This validation experiment should result in a standard deviation ≤0.10 for all probes over the experiment.

Required specimens

Extracted DNA from human peripheral whole blood specimens, buccal swab, (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, foetal blood, or products of conception free from maternal contamination,

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
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 without intellectual disability, developmental delay and/or congenital abnormalities. 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 NA06047, NA22991, NA14131 and NA00501 from the Coriell Institute have been tested with this P070-B3 probemix at MRC Holland and can be used as a positive control samples to detect CNVs in subtelomeric regions, see table below for more detailed information. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Chromosomal position of copy number alteration*	Probes affected	Expected copy number alteration
NA06047	Coriell Institute	17p	RPH3AL	Heterozygous deletion
NA22991	Coriell Institute	1p	TNFRSF18	Heterozygous deletion
NA14131	Coriell Institute	5p	CCDC127	Heterozygous deletion
NA00501	Coriell Institute	2p and 4q	ACP1 and FRG1	Heterozygous deletion of 2p Heterozygous duplication of 4q

* Indicated chromosomal bands accommodate genes targeted by MLPA probes, however, the whole extent of copy number alteration (CNA) present in this cell line cannot be determined by this P070-B3 Subtelomeres Mix 2B probemix.

Performance characteristics
The exact performance characteristics cannot easily be determined due to the difficulty in obtaining sufficient DNA samples containing deletions or duplications for all probes. Ahn et al. (2007) determined 95% confidence intervals for positive and negative predictive accuracies to be 0.951-0.996 and 0.9996-1 respectively in their laboratory. In general, each positive result needs to be confirmed and further studied.

In postnatal samples from patients with non-syndromic intellectual disability and/or developmental delay, the P036 and P070 subtelomere probemixes detect subtelomeric CNVs in approximately 3-6% of the cases. However, the diagnostic sensitivity depends highly on the inclusion criteria of the patients that were tested. When patients suffering from non-syndromic intellectual disability, developmental delay and congenital abnormalities are included, CNVs are found in approximately 10% of the cases. For prenatal samples, the diagnostic sensitivity is 0.5-4% and the diagnostic sensitivity for products of conception (POC) is 18-62%.

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 allele copy number for all probes, including the probes for the pseudo-autosomal regions of the sex chromosomes, are 2 (normal), 1 (heterozygous deletion) or 3 (heterozygous duplication). The standard deviation of each individual probe over all the reference samples should be ≤0.10. When this criterium is fulfilled, the following cut-off values for the final ratio (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.

- 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.
- False results can be obtained if one or more peaks are off-scale. 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.
- Interpretation of abnormal copy number findings in subtelomeric regions is complicated. Subtelomeric copy number changes can also occur in unaffected individuals and the effect of a deletion or duplication will depend on the genes involved. A considerable number of abnormalities detected by a single probe may not be the cause of any phenotypic effect but can be due to a rare polymorphism or a copy number change which is also present in one of the parents. For some chromosome arms, even large subtelomeric deletions or duplications (>1 Mb) can be inherited without a clear phenotypic effect. For all abnormalities detected, we strongly recommend testing parents to determine whether the copy number aberration in the patient is de novo.
- 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.

P070 specific notes:

- The pathogenic phenotypes caused by subtelomeric deletions or duplications are in most cases autosomal dominant disorders. Copy number changes of a single chromosome can be pathogenic.

- Deletions can result in haploinsufficiency of one or more genes. Duplications can lead to distortion of gene expression or can result in haploinsufficiency of genes that are disrupted by the duplication breakpoints. Analysis of parental samples may be necessary for correct interpretation of results.
- For each subtelomeric region, the copy number of only a single small (approximately 70 nt) sequence is determined. **These sequences have been selected on the basis of their proximity to the telomere and are usually located in genes that have not been directly implicated in intellectual disability, developmental delay and/or congenital abnormalities.** For many chromosomes, it is not known what minimum size is required for a subtelomeric deletion/duplication to have pathogenic effects.
- Most probes in the P036 and P070 probemixes target well-characterised genes at short distance of the telomere. An exception is made for the p-arms of chromosomes 13, 14, 15, 21 and 22 as these are covered by over 10 Mb of repeat sequences. Here, the "p" probes recognise one of the first genes on the q arm, close to centromere.

Limitations of the procedure

- 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.
- For use on (un)cultured amniocytes and (un)cultured chorionic villi, 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).

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.

False negative results can occur given that each chromosomal arm is covered by a single probe; whereas false positive results may be due to polymorphisms or experimental variation. Given the high chance of false positive/negative results, it is recommended to use both MLPA probemix P036 and MLPA probemix P070 for initial testing of each DNA sample. **All P070 probes differ from P036 probes.** Copy number changes detected by P036 **and** P070 may still be without clinical significance. In case of positive results, it is therefore strongly recommended to also test the parents. **In case P036 and P070 give conflicting results, follow-up studies are needed.**

To confirm and delineate the extent of a subtelomeric deletion/duplication, MLPA follow-up probemixes containing multiple probes for a particular subtelomeric region are available, see related probemixes and/or www.mrcholland.com. Confirmation may also be done by FISH, array CGH or NGS. Confirmation of results may be difficult: deletions/duplications can be interstitial and may not extend to the telomere. P036 and P070 probes may detect sequences which are located quite far from each other or from the targets of commercially available FISH probes.

In case of suspected aneuploidy in which probes of the q-arm and p-arm (or q-cen for acrocentric chromosomes 13, 14, 15, 21 and 22) of a specific chromosome are affected in the same way, confirmation

should be done by alternative methods, or follow-up MLPA probemix P095 can be used to detect aneuploidies of chromosomes 13, 18, 21, X and Y.

Database of Genomic variants and Phenotype
http://decipher.sanger.ac.uk/. We strongly encourage users to deposit positive results in the Decipher database.

Please report false positive results due to SNVs and other unusual results to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P070-B3 Subtelomeres Mix 2B

Length (nt)	SALSA MLPA probe	Gene detected	Chromosomal position (hg18)	Location (hg18) in kb
64-121	Control fragments – see table in probemix content section for more information			
132	04084-L03605	SH3BP5L	1q	01-247,078 (0.2 Mb from telomere)
139	02781-L03168	ATG4B	2q	02-242,247 (0.7 Mb from telomere)
145	02690-L02842	KIAA0226	3q	03-198,884 (0.6 Mb from telomere)
152 ±	02691-L02843	FRG1	4q	04-191,099 (0.2 Mb from telomere)
160	02790-L02232	GNB2L1	5q	05-180,602 (0.3 Mb from telomere)
166 ±	02694-L02844	TBP	6q	06-170,708 (0.2 Mb from telomere)
172	02793-L03167	VIPR2	7q	07-158,628 (0.2 Mb from telomere)
179 «	02695-L00610	RECQL4	8q	08-145,709 (0.6 Mb from telomere)
186	02792-L02846	EHMT1	9q	09-139,777 (0.5 Mb from telomere)
193 «	02696-L02847	ECHS1	10q	10-135,026 (0.3 Mb from telomere)
202	02697-L02848	IGSF9B	11q	11-133,293 (1.2 Mb from telomere)
211	02686-L02849	ZNF10	12q	12-132,242 (0.1 Mb from telomere)
218	02698-L00753	CDC16	13q	13-114,027 (0.1 Mb from telomere)
226	02699-L02850	MTA1	14q	14-104,992 (1.4 Mb from telomere)
233	18224-L23503	TM2D3	15q	15-100,008 (0.3 Mb from telomere)
241	02702-L00734	GAS8	16q	16-088,638 (0.2 Mb from telomere)
250	02703-L03169	SECTM1	17q	17-077,874 (0.9 Mb from telomere)
257	02704-L03607	CTDP1	18q	18-075,576 (0.5 Mb from telomere)
265 «	02705-L02853	CHMP2A	19q	19-063,755 (0.1 Mb from telomere)
274	02706-L00642	UCKL1	20q	20-062,046 (0.4 Mb from telomere)
281	02587-L02854	S100B	21q	21-046,847 (0.1 Mb from telomere)
290	02707-L00661	ARSA	22q	22-049,413 (0.3 Mb from telomere)
298	02708-L02855	VAMP7	X/Yq (PAR2)	X-154,826 + Y-057,685 (PAR2; both 0.1 Mb from telomere)
306 «	02270-L01762	TNFRSF18	1p	01-001,129
315	02709-L02856	ACP1	2p	02-000,267
323	02896-L02363	CHL1	3p	03-000,336
329	14440-L16146	PIGG	4p	04-000,506
337	02791-L02233	CCDC127	5p	05-000,259
346	04077-L03462	IRF4	6p	06-000,340
355	02780-L02857	SUN1	7p	07-000,845
362	02715-L00973	FBXO25	8p	08-000,398
370	02716-L00688	DOCK8	9p	09-000,376
379	05180-L16343	ZMYND11	10p	10-000,216
387	02784-L02226	BET1L	11p	11-000,195
393	02787-L02229	KDM5A	12p	12-000,287
402 + «	02717-L27468	PSPC1	13q-cen	13-019,255 (Acrocentric)
409 +	02718-L27469	PARP2	14q-cen	14-019,896 (Acrocentric)
418 + «	04026-L01542	NDN	15q-cen	15-021,482 (Acrocentric)
427 «	02720-L00648	DECR2	16p	16-000,402
436	04081-L03465	RPH3AL	17p	17-000,183

444	02789-L02231	THOC1	18p	18-000,205
451 ±	03501-L23504	PPAP2C	19p	19-000,232
459	02723-L00641	ZCCHC3	20p	20-000,227
466 +	02724-L00334	HSPA13	21q-cen	21-014,675 (Acrocentric)
479 +	02725-L16344	IL17RA	22q-cen	22-015,960 (Acrocentric)
490	03714-L27631	SHOX	X/Yp (PAR1)	X/Y-000,522 (PAR1 region)

± SNP rs189426000 could influence the 152 probe; rs573618262 could influence the 166 probe and rs34708574 could influence the 451 probe. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
« 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.
+ The 13, 14, 15, 21 & 22 q-cen probes target the q-arm close to the centromere (acrocentric chromosomes), as the p-arm of these chromosome do not contain well-characterised genes.

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 table above.

Important notes on specific subtelomeres
Several probes are located in regions that show copy number variation in healthy individuals, see http://dgv.tcag.ca/dgv/app/home. Therefore, we strongly recommend ALWAYS confirming results by a designated MLPA follow-up probemix or other suitable method.

Please inform us about any possible improvements or interesting findings: info@mrcholland.com.

- **1p**: The **P070** probe detects a sequence that was found to be duplicated in a healthy individual.
- **3p**: The **P036/P070** probes detect sequences that were found to be duplicated in healthy individuals. According to Dijkhuizen et al. (2006), defects in the more centromeric *CNTN4* and *CRBN* genes might be more important for the 3p syndrome than *CHL1* loss.
- **12p**: The **P036/P070** probes detect sequences that were found to be duplicated in healthy individuals.
- **15q-cen**: The **P036/P070** probes detect sequences that were found deleted in some Prader-Willi/Angelman syndrome (PWS/AS) patients. We recommend SALSA MLPA Probemix ME028 Prader-Willi/Angelman (Research use only) for further characterisation of the 15q11-13 region.
- **16p**: The **P036/P070** probes are variable. P036 and P070 probes target sequences that are 0.36 Mb separated and, therefore, might not always be able to confirm each other. The SALSA MLPA Probemix P140 HBA (IVD) contains multiple probes on the 16p subtelomere region and can be used to further characterise 16p deletions and duplications.
- **20p**: The **P036/P070** probes detect sequences that were found to be duplicated in healthy individuals.
- **3q**: The **P036** probe for 3q (*BDH1* gene) was found to be duplicated in a healthy parent by E. Reyniers (Medical Genetics, University of Antwerp), this was confirmed by FISH. The **P070** probe for 3q, located 200 kb closer to the telomere, did not show this duplication. Apparently, CNVs of the *BDH1* gene region can occur in healthy individuals. However, please note that a 3q29 microdeletion syndrome (due to an interstitial deletion) has been described (Willatt et al. 2005) and that the probe in the **P036** probemix detects a sequence within this commonly deleted region. The 3q probe in **P070** will not detect this microdeletion, as it detects a sequence located between the interstitial deletion and the telomere.
- **4q**: The **P036/P070** probes detect the complex 4q telomeric region in which very few genes are present. The *FRG1* gene is the only well-characterised gene in the terminal 2-3 Mb of 4q, but most *FRG1*-specific probes tested were found to be unreliable due to the presence of population-specific SNPs in *FRG1*. The 4q probe in **P036** is located at a larger distance from the telomere; we do not expect this to result in false-negatives as deletions of the telomeric 2-3Mb region of 4q are not associated with any phenotypic effects (Shao et al. 2008).
- **5q**: The **P036** probe for 5q was affected in one patient due to a polymorphism in the first nucleotide after the ligation site. The **P036/P070** probes for 5q detect sequences that have been found to be duplicated sporadically in healthy individuals.
- **12q**: The **P036/P070** probes detect sequences that were found to be duplicated in a healthy individual.
- **19q**: The **P036/P070** probes detect sequences that have variable copy numbers in healthy individuals.
- **22q**: The **P070** probe detects a sequence that was found to be deleted in healthy individuals. The sequence detected by the **P036** probe for 22q has only one mismatch with a related sequence on chromosome 2. This is sufficient to generate a chr. 22q specific MLPA signal. However, when this related region is to be sequenced, primer design is complicated as only 4 mismatches are present in the 350 nt region containing

this probe sequence. The SALSA MLPA Probemix P188 22q13 (Research use only) contains many probes close to the 22q13 telomere and can be used to confirm or further characterise 22q CNVs.
- **X, Y**: The sex chromosome specific probes in **P036/P070** detect sequences located on both X and Y chromosome close to the telomeres (PAR region) and will thus detect the combined copy number of X and Y.

SALSA MLPA probemixes P036 Subtelomeres Mix 1 and P070 Subtelomeres Mix 2B
Probemixes *P036 Subtelomeres Mix 1* and *P070 Subtelomeres Mix 2B* both contain one probe for each subtelomere and can be used to detect most deletions/duplications in the subtelomeric regions. Using both probemixes together maximises the detection rate and reduces the risk of false positives due to polymorphisms affecting a single probe.. We recommend following the strategy outlined in Figure 1.

When used correctly, screening with MLPA probemixes P036 and P070 will detect or exclude the presence of abnormal copy numbers of subtelomeric regions in the majority of samples. MLPA will however not detect inversions or balanced translocations. Interpretation of abnormal copy number findings in subtelomeric regions is complicated. Subtelomeric copy number changes also occur in healthy individuals and the effect of a deletion or duplication will depend on the genes involved. The Database of Genomic Variants can be useful to verify whether copy number changes occur in normal individuals: http://dgv.tcag.ca/dgv/app/home.

A considerable number of abnormalities detected by a single probe may not be the cause of any phenotypic effect but can be due to a rare polymorphism. When abnormalities are detected using P036/P070, these findings should always be confirmed by an MLPA probemix for a specific syndrome (see www.mrcholland.com) or another technique (e.g. FISH, array-CGH).

Finding the genetic cause of congenital abnormalities with MLPA probemix P036 and P070
The number of genes associated with intellectual disability and/or developmental delay is large. In some cases, particular phenotypic features suggest the involvement of a specific gene or chromosomal region. Numerous SALSA MLPA probemixes are available to find the cause of developmental delay with distinct syndromic features, such as RETT, Sotos and Prader-Willi/Angelman syndrome.

For individuals with (suspicion of) intellectual disability or developmental delay and/or congenital abnormalities lacking distinct syndromic features, the genetic cause is found only in a minority of cases. When using the P036 and P070 probemixes, we suggest following the strategy as outlined in Figure 1.

When **no abnormalities** are detected using P036/P070, the follow-up SALSA MLPA Probemix P245 Microdeletion Syndromes-1A (IVD) can be used for screening for distinct microdeletion syndromes (see P245 product description for details). Numerous other probemixes can be found online at www.mrcholland.com and, several probemixes are highlighted below in the Related SALSA MLPA probemixes section.

Finding the genetic cause of pregnancy loss with MLPA
The genetic cause of pregnancy loss can be found using P036/P070 on POC samples. Approximately 50-60% of first trimester spontaneous miscarriages are caused by chromosomal abnormalities, of which the vast majority is aneuploid (Wu T et al., 2019). In case of suspected aneuploidy, follow up probemixes or alternative methods are needed to confirm the results. We suggest following the strategy as outlined in Figure 1 below.

Prenatal diagnosis with MLPA
Using P036 and P070 for prenatal testing is recommended when there is an increased risk for intellectual disability, e.g. in high-risk pregnancies or when there is a suspicion of abnormalities, e.g. after ultrasound findings. We suggest following the strategy as outlined in Figure 1.

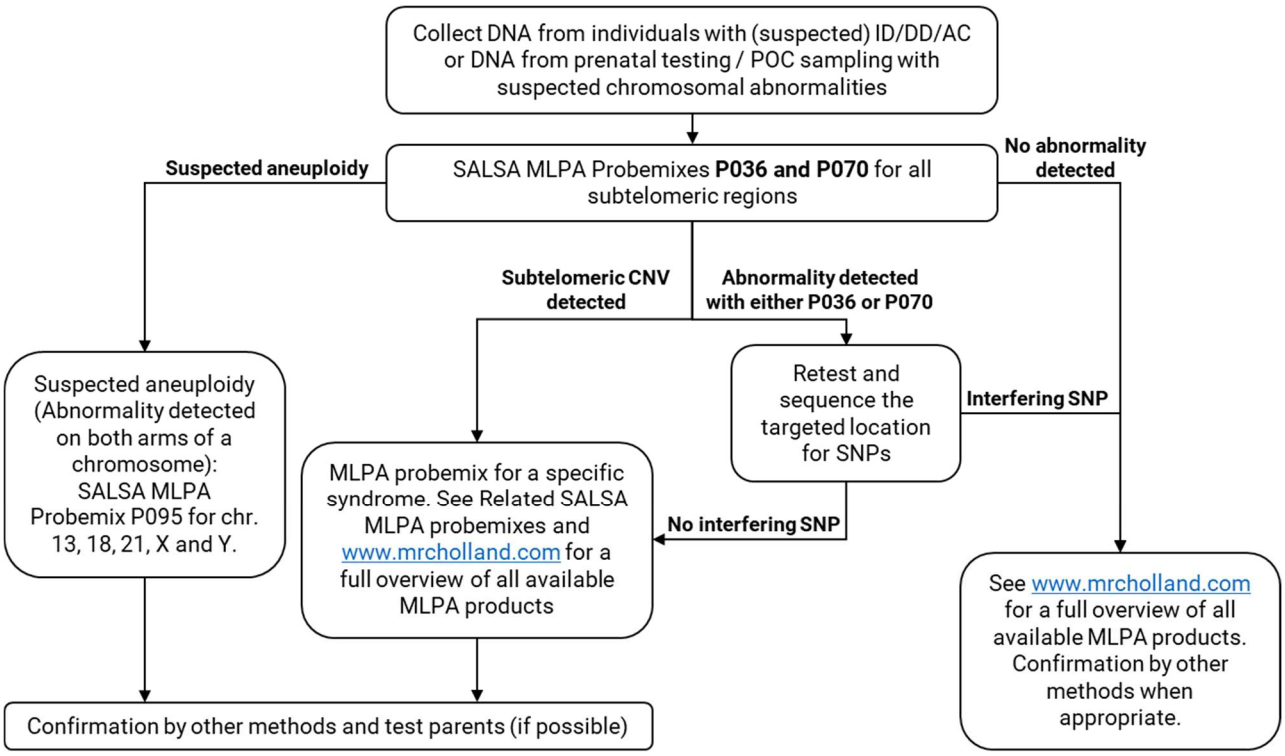

Figure 1: Flowchart suggesting how to test an individual with suspicion of ID/DD/CA lacking clear syndromic features and, prenatal testing after higher risk for chromosomal abnormalities or POC testing. ID/DD/CA: intellectual disability/ developmental delay/ congenital abnormalities.

Related SALSA MLPA probemixes

P036 Subtelomeres Mix 1	Contains one probe for each of the 41 subtelomeric regions and 5 probes near the centromeric regions of the five acrocentric chromosomes. All probes differ from the P070 probes. We strongly recommend using both P036 and P070 on each sample.
P245 Microdeletion Syndromes-1A	Probes for 23 different microdeletion syndromes; can be used for primary screening of microdeletion syndromes.
P064 Microdeletion Syndromes-1B	Probes for 15 different microdeletion syndromes; can be used for primary screening of microdeletion syndromes.
P297 Microdeletion Syndromes-2	Probes for 11 different microdeletion syndromes; can be used for primary screening of microdeletion syndromes.
P106 X-linked ID	Probes for several genes involved in X-linked mental retardation.
P095 Aneuploidy	Probes for chromosomes 13, 18, 21, X and Y to detect aneuploidy of these chromosomes.
P181 Centromere-1	Contains one probe for each of 41 different centromeric regions and an extra probe near the centromeric regions of each of the five acrocentric chromosomes, resulting in two probes for each chromosome.
P182 Centromere-2	Similar to P181 centromeres but all probes are different from the P181 probes.
P147 1p36	Probes for the 1p36 subtelomeric region.
P140 HBA	Probes for the human alpha-globin (HBA) gene cluster and its regulatory region located on chromosome 16p13.3, as a potential cause for and clinical diagnosis of alpha-thalassaemia.
P188 22q13	Probes for the 22q12 and 22q13 chromosomal regions (research use only). A partial 22q13 deletion is associated with the Phelan-McDermid syndrome.
ME028 Prader-Willi/Angelman	Probes to detect an aberrant methylation of one or more sequences of the 15q11 chromosomal region, as well as copy number changes in this chromosomal region (research use only).

More probemixes are available for specific syndromes, including RETT(-like) syndrome, DiGeorge, Canavan, Lissencephaly, Williams syndrome and many more (see www.mrcholland.com).

References

- Ahn JW et al. (2007). Detection of subtelomere imbalance using MLPA: validation, development of an analysis protocol, and application in a diagnostic centre. *BMC medical genetics*. 8:9.
- Bernatowicz, K., et al., Clinical Utility of MLPA and QF-PCR Techniques in the Genetic Testing of Miscarriages. *Russ. J. Genet.* 2019. 55(10): 1259-1265.
- Castells-Sarret, N., et al., (2017) Comparative genomic hybridisation as a first option in genetic diagnosis: 1000 cases and a cost–benefit analysis. *An Pediatr* (English Edition). 89(1): 3-11.
- Dijkhuizen T et al. (2006). FISH and array-CGH analysis of a complex chromosome 3 aberration suggests that loss of CNTN4 and CRBN contributes to mental retardation in 3pter deletions. *Am J Med Genet A.* 140:2482-2487.
- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Shao L et al. (2008). Identification of chromosome abnormalities in subtelomeric regions by microarray analysis: a study of 5,380 cases. *Am J Med Genet A.* 146A:2242-2251.
- van den Berg C et al. (2006). (Potential) false-negative diagnoses in chorionic villi and a review of the literature. *Prenat Diagn.* 26:401-408.
- Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem*. 421:799-801.
- Willatt L et al. (2005). 3q29 microdeletion syndrome: clinical and molecular characterization of a new syndrome. *Am J Hum Genet.* 77:154-160.
- Wu T et al. (2019). Evaluation of two aneuploidy screening tests for chorionic villus samples: Multiplex ligation-dependent probe amplification and fluorescence in situ hybridization. *Mol Cell Probes*. 46:101422.

Selected publications using SALSA MLPA Probemix P070 Subtelomeres Mix 2B

- Ahn JW et al. (2008). Submicroscopic chromosome imbalance in patients with developmental delay and/or dysmorphism referred specifically for Fragile X testing and karyotype analysis. *Mol. Cytogenet.* 1:2.
- Bendavid C et al. (2007). MLPA screening reveals novel subtelomeric rearrangements in holoprosencephaly. *Hum. Mutat.* 28:1189-1197.
- Brosens E et al. (2014). Structural and numerical changes of chromosome X in patients with esophageal atresia. *Eur. J. Hum.* 22:1077-1084.
- Bruno DL et al. (2006). High-throughput analysis of chromosome abnormality in spontaneous miscarriage using an MLPA subtelomere assay with an ancillary FISH test for polyploidy. *Am J Med Genet A.* 140:2786-2793.
- Capkova P et al. (2019). MLPA is a practical and complementary alternative to CMA for diagnostic testing in patients with autism spectrum disorders and identifying new candidate CNVs associated with autism. *PeerJ.* 6:e6183.
- Ceroni JRM et al. (2018). A Multicentric Brazilian Investigative Study of Copy Number Variations in Patients with Congenital Anomalies and Intellectual Disability. *Sci Rep*. 8:13382.
- Christofolini DM et al. (2010). Subtelomeric rearrangements and copy number variations in people with intellectual disabilities. *J. Intellect. Disabil.* 54:938-942.
- Damnjanovic T et al. (2015). Subtelomeric screening in Serbian children with dysmorphic features and unexplained developmental delay/intellectual disabilities. *Turk. J. Pediatr.* 57:154-160.
- Delahaye A et al. (2009). Chromosome 22q13.3 deletion syndrome with a de novo interstitial 22q13.3 cryptic deletion disrupting SHANK3. *Eur. J. Med. Genet.* 52:328-332.
- Diego-Alvarez D et al. (2007). MLPA as a screening method of aneuploidy and unbalanced chromosomal rearrangements in spontaneous miscarriages. *Prenat. Diagn.* 27:765-771.
- Donaghue C et al. (2010). Combined QF-PCR and MLPA molecular analysis of miscarriage products: an efficient and robust alternative to karyotype analysis. *Prenat. Diagn.* 30:133-137.
- Erjavec-Skerget A et al. (2006). Subtelomeric chromosome rearrangements in children with idiopathic mental retardation: applicability of three molecular-cytogenetic methods. *Croat. Med. J.* 47:841-850.

- Faas BH et al. (2008). Detection of cryptic subtelomeric imbalances in fetuses with ultrasound abnormalities. *Eur. J. Med. Genet.* 51:511-519.
- Grati FR et al. (2008). Prenatal detection by subtelomeric FISH and MLPA of unbalanced meiotic recombinants resulting from parental pericentric inversions. *Mol. Cell. Probes.* 22:316-319.
- Hila L et al. (2009). MLPA subtelomere analysis in Tunisian mentally retarded patients. *Biochem. Genet.* 47:727-733.
- Isidori I et al. (2017). QF-PCR and MLPA: a reliable molecular system to detect chromosomal alterations in miscarriages. *Clin Exp Obstet Gynecol.* 44:220-225.
- Kim JW et al. (2015). Molecular analysis of miscarriage products using multiplex ligation-dependent probe amplification (MLPA): alternative to conventional karyotype analysis. *Arch. Gynecol. Obstet.* 291:347-354.
- Konialis C et al. (2011). Uncovering recurrent microdeletion syndromes and subtelomeric deletions/duplications through non-selective application of a MLPA-based extended prenatal panel in routine prenatal diagnosis. *Prenat. Diagn.* 31:571-577.
- Koolen DA et al. (2004). Screening for subtelomeric rearrangements in 210 patients with unexplained mental retardation using multiplex ligation dependent probe amplification (MLPA). *J. Med. Genet.* 41:892-899.
- Lam AC et al. (2006). High rate of detection of subtelomeric aberration by using combined MLPA and subtelomeric FISH approach in patients with moderate to severe mental retardation. *Clin. Biochem.* 39:196-202.
- Mademont-Soler I et al. (2010). Subtelomeric MLPA: is it really useful in prenatal diagnosis? *Prenat. Diagn.* 30:1165-1169.
- Mandal K et al. (2009). Use of Multiplex Ligation-Dependent Probe Amplification (MLPA) in screening of subtelomeric regions in children with idiopathic mental retardation. *Indian J. Pediatr.* 76:1027-1031.
- Medina A et al. (2014). Multiplex ligation-dependent probe amplification to subtelomeric rearrangements in idiopathic intellectual disability in Colombia. *Pediatr. Neurol.* 50:250-254.
- Mohan S et al. (2016). Genomic imbalance in subjects with idiopathic intellectual disability detected by multiplex ligation-dependent probe amplification. *Journal of genetics.* 95:469-474.
- Monfort S et al. (2006). Evaluation of MLPA for the detection of cryptic subtelomeric rearrangements. *J Lab Clin Med.* 147:295-300.
- Mundhofir FE et al. (2013). Subtelomeric chromosomal rearrangements in a large cohort of unexplained intellectually disabled individuals in Indonesia: A clinical and molecular study. *Indian J. Hum. Gene.* 19:171-178.
- Northrop EL et al. (2005). Detection of cryptic subtelomeric chromosome abnormalities and identification of anonymous chromatin using a quantitative multiplex ligation-dependent probe amplification (MLPA) assay. *Human mutation.* 26:477-486.
- Novo-Filho GM et al. (2016). Subtelomeric Copy Number Variations: The Importance of 4p/4q Deletions in Patients with Congenital Anomalies and Developmental Disability. *Cytogenet. Genome Res.* 149:241-246.
- Palomares M et al. (2006). MLPA vs multiprobe FISH: comparison of two methods for the screening of subtelomeric rearrangements in 50 patients with idiopathic mental retardation. *Clin. Genet.* 69:228-233.
- Rooms L et al. (2006). Multiplex ligation-dependent probe amplification to detect subtelomeric rearrangements in routine diagnostics. *Clin. Genet.* 69:58-64.
- Rosello M et al. (2010). Prenatal study of common submicroscopic "genomic disorders" using MLPA with subtelomeric/microdeletion syndrome probe mixes, among gestations with ultrasound abnormalities in the first trimester. *Eur. J. Med. Genet.* 53:76-79.
- Ruiter EM et al. (2007). Pure subtelomeric microduplications as a cause of mental retardation. *Clin. Genet.* 72:362-368.
- Saxena D et al. (2016). Utility and limitations of multiplex ligation-dependent probe amplification technique in the detection of cytogenetic abnormalities in products of conception. *J. Postgrad. Med.* 62:239-241.
- Stegmann AP et al. (2008). Prospective screening of patients with unexplained mental retardation using subtelomeric MLPA strongly increases the detection rate of cryptic unbalanced chromosomal rearrangements. *Eur. J. Med. Genet.* 51:93-105.

- Stevens SJ et al. (2009). Identical cryptic partial monosomy 20pter and trisomy 20qter in three adult siblings due to a large maternal pericentric inversion: detection by MLPA and breakpoint mapping by SNP array analysis. *Am J Med Genet A.* 149A:2226-2230.
- Van Opstal D et al. (2011). Multiplex ligation dependent probe amplification (MLPA) for rapid distinction between unique sequence positive and negative marker chromosomes in prenatal diagnosis. *Mol. Cytogenet.* 4:2.
- Wu T et al. (2019). Evaluation of two aneuploidy screening tests for chorionic villus samples: Multiplex ligation-dependent probe amplification and fluorescence in situ hybridization. *Mol Cell Probes.* 46:101422.
- Wu Y et al. (2010). Submicroscopic subtelomeric aberrations in Chinese patients with unexplained developmental delay/mental retardation. *BMC Med. Genet.*. 11:72.
- Zanardo EA et al. (2017). Cytogenomic assessment of the diagnosis of 93 patients with developmental delay and multiple congenital abnormalities: The Brazilian experience. *Clinics (Sao Paulo)*. 72:526-537.
- Zimowski JG et al. (2016). First-trimester spontaneous pregnancy loss - molecular analysis using multiplex ligation-dependent probe amplification. *Clin. Genet.* 89:620-624.

P070 Product history	
Version	Modification
B3	118 nt Y chromosome control fragment replaced; 5 other probes a small change in length.
B2	88 & 96 nt DNA Denaturation control fragments replaced (QDX2).
B1	New 4p probe at 329 nt added; 3 other probes have a change in length.
A2	Extra control fragments at 88-96-100 and 105 nt added (QDX1).
A2	No change compared to previous lot (0407).
A1	New probes at 1q, 6p, "15p", 17p, X/Yp; size change for 1 probe.
A	First release.

Implemented changes in the product description

Version B3-05 – 25 March 2021 (04P)
- Product description rewritten and adapted to a new template.
- Intended purpose updated to include the recommendation of using P070 together with P036 and adapted to a new template.
- Clinical background information rewritten.
- Positive DNA sample information is updated.
- Table 1 updated with new insights (e.g. SNPs and salt sensitive probes), probe location in kb and distance to telomere updated according to hg18.
- Important notes paragraph updated with new insights, added Probemix P140 to confirm 16p.
- P036/P070 paragraph rewritten and expanded to include prenatal and POC samples.
- Figure 1 updated and expanded to include prenatal and POC samples.
- SALSA MLPA probemixes P095, P297, P140, P188 and ME028 added to the related probemixes.
- The sections References and Selected publications are updated.
- UK has been added to the list of countries in Europe that accept the CE mark.

Version B3-04 – 17 June 2020 (04)
- Colombia and Israel added as countries with IVD status.

Version B3-03 – 05 June 2018 (04)
- Product description adapted to a new template.
- Information regarding probemix P069 and the telomere follow-up probemixes has been removed.
- IVD use now includes Morocco.

Version B3-02 – 14 December 2017 (03)
- Products of conception has been added to the intended use.
- SALSA Artificial Duplication DNA SD025 is obsolete and information regarding SD025 has been removed.

- Note on page 3 added: In some instances the nearest gene is mentioned (table 1) as the gene that is detected by a particular probe.
- Table 1: 306 and 427 nt probes were marked as salt sensitive.
- "p" probes on chromosomes 13, 14, 15, 21, 22 were renamed "q-cen" to align with P036 product description.
- Related Probemixes: P245 and P064 renamed to Microdeletion Syndromes 1A and 1B, respectively.
- References added to selected publications.

Version B3-01 – 28 November 2016 (03)

- Product description restructured and adapted to a new template.
- Updated intended use and required specimens.
- Added section on important notes on specific subtelomeres.

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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*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.