Product Description
SALSA® MLPA® Probemix P057-B3 FANCD2-PALB2

To be used with the MLPA General Protocol.

Version B3
As compared to version B2, five reference probes have been replaced and several probes have a change in length but no change in the sequence targeted. For complete product history see page 7.

Catalogue numbers:
- **P057-025R:** SALSA MLPA Probemix P057 FANCD2-PALB2, 25 reactions.
- **P057-050R:** SALSA MLPA Probemix P057 FANCD2-PALB2, 50 reactions.
- **P057-100R:** SALSA MLPA Probemix P057 FANCD2-PALB2, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit and Coffalyser.Net data analysis software. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman/SCIEX capillary sequencers, respectively (see www.mrcholland.com).

Certificate of Analysis
Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mrcholland.com.

Precautions and warnings
For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mrcholland.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions from findings generated with this product.

General information
The SALSA MLPA Probemix P057 FANCD2-PALB2 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *FANCD2* and *PALB2* genes, which are associated with Fanconi Anemia (FA).

FA is an autosomal recessive disorder affecting all bone marrow elements and associated with cardiac, renal, and limb malformations as well as with dermal pigmentary changes. Several FA-associated genes have been identified so far, the products of which function in the FA/BRCA pathway. A key event in the pathway is the monoubiquitination of the FANCD2 protein, which depends on a multiprotein FA core complex. Defects in the *FANCD2* gene are one of the possible causes of FA. The *FANCD2* gene (44 exons) spans ~73 kb of genomic DNA and is located on chromosome 3p25.3, about 10 Mb from the p-telomere.

FA has also been linked to defects in the *PALB2* gene. It was shown that mutations in *PALB2* result in an increased susceptibility to breast cancer, that biallelic mutations cause Fanconi anemia subtype FA-N, and predispose to childhood cancers. *PALB2* mutations have also been detected in approximately 3% of familial pancreatic cancer families, especially those families in which also breast cancer cases occur (Slater et al. 2010). The *PALB2* gene spans ~38 kb of genomic DNA and is located on chromosome 16p12.1, about 24 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1401/.

This SALSA MLPA probemix is not CE/FDA registered for use in diagnostic procedures. Purchase of this product includes a limited license for research purposes.

Gene structure and transcript variants:
Entrez Gene shows transcript variants of each gene: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
For NM_ mRNA reference sequences: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Exon numbering
The *FANCD2* and *PALB2* exon numbering used in this P057-B3 FANCD2-PALB2 product description is the exon numbering from the LRG_306 and LRG_308 sequences, respectively. The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the 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 P057-B3 FANCD2-PALB2 contains 39 MLPA probes with amplification products between 130 and 431 nucleotides (nt). This includes 15 probes for the *FANCD2* gene, targeting 14 out of 44 exons, and 13 probes for the *PALB2* gene, one probe for each exon. In addition, 11 reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

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

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

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

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

Required specimens
Extracted DNA 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 Fanconi Anemia. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol (www.mrcholland.com).

Positive control DNA samples
MRC Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (https://catalog.coriell.org) and Leibniz Institute DSMZ (https://www.dsmz.de/) have diverse collections of biological resources which may be used as positive

control DNA samples in your MLPA experiments. The quality of cell lines can change; therefore samples should be validated before use.

Data analysis

Coffalyser.Net software should be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net software is freely downloadable at www.mrcholland.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

Interpretation of results

The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the final ratio (FR) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the following cut-off values for the FR of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	1.75 < FR < 2.15
Ambiguous copy number	All other values

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

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net

software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *FANCD2* and *PALB2* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P057 FANCD2-PALB2.
- MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can cause false positive results. Mutations/SNVs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.

Confirmation of results

The P260 PALB2-RAD50-RAD51C-RAD51D can be used for confirmation of results for seven *PALB2* probes of the P057 (see Table 2b). The other six *PALB2* probes cannot be confirmed by probemix P260 because their ligation site is similar or close to the probes in the P057.

Copy number changes detected by only a single probe always require confirmation by another method. An apparent deletion detected by a single probe can be due to e.g. a mutation/polymorphism that prevents ligation or destabilises the binding of probe oligonucleotides to the DNA sample. Sequence analysis can establish whether mutations or polymorphisms are present in the probe target sequence. The finding of a heterozygous mutation or polymorphism indicates that two different alleles of the sequence are present in the sample DNA and that a false positive MLPA result was obtained.

Copy number changes detected by more than one consecutive probe should be confirmed by another independent technique such as long range PCR, qPCR, array CGH or Southern blotting, whenever possible. Deletions/duplications of more than 50 kb in length can often be confirmed by FISH.

Fanconi Anemia mutation database

http://www2.rockefeller.edu/fanconi/. We strongly encourage users to deposit positive results in the Fanconi Anemia mutation database of the Rockefeller University. 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 *PALB2* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P057-B3 FANCD2-PALB2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*FANCD2*	*PALB2*
64-105	Control fragments – see table in probemix content section for more information			
130 ¥	Reference probe 18709-L24593	5q		
142	**PALB2 probe** 07492-L09488			**Exon 1**
150 *	Reference probe 10301-L30389	11q		
156 ¥	**PALB2 probe** 07933-L32646			**Exon 9**
161	**PALB2 probe** 16145-L18314			**Exon 12**
166	**PALB2 probe** 07501-L07163			**Exon 10**
172	**FANCD2 probe** 02129-L07570		**Exon 2**	
178	**FANCD2 probe** 02130-L07571		**Exon 4**	
184 *	Reference probe 10904-L27810	9q		
193	**FANCD2 probe** 02131-L01624		**Exon 9**	
200	Reference probe 16990-L19999	17q		
208	**PALB2 probe** 16146-L18315			**Exon 7**
214 Δ	**FANCD2 probe** 02132-L28606		**Exon 12**	
220	**FANCD2 probe** 16147-L18316		**Exon 30**	
226	Reference probe 10240-L04097	9q		
232	**FANCD2 probe** 16148-L18317		**Exon 6**	
240	Reference probe 17009-L20056	1q		
249	**FANCD2 probe** 16149-L28607		**Exon 10**	
256	**FANCD2 probe** 16150-L18319		**Exon 29**	
265	**FANCD2 probe** 01649-L01226		**Exon 1**	
274	**FANCD2 probe** 02137-L01630		**Exon 32**	
283	**FANCD2 probe** 02138-L01631		**Exon 35**	
292	**PALB2 probe** 07502-L07164			**Exon 11**
301	**FANCD2 probe** 02139-L19933		**Exon 41**	
310	**FANCD2 probe** 02140-L01633		**Exon 43a**	
319	Reference probe 01042-L10915	8q		
330	**FANCD2 probe** 01650-L19934		**Exon 1**	
337	**PALB2 probe** 07504-L07166			**Exon 13**
344 *	Reference probe 12785-L27941	2q		
355	**PALB2 probe** 07495-L28608			**Exon 4**
364	**FANCD2 probe** 16151-L18320		**Exon 38**	
373	**PALB2 probe** 07497-L07159			**Exon 6**
384	**PALB2 probe** 16152-L18321			**Exon 8**
391 *	Reference probe 20292-L31436	14q		
401	**PALB2 probe** 07494-L07156			**Exon 3**
409	**PALB2 probe** 07496-L06744			**Exon 5**
418 *	Reference probe 18479-L23656	6q		
427	**PALB2 probe** 07493-L28609			**Exon 2**
431	Reference probe 15541-L25346	2q		

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

* New in version B3.
¥ Changed in version B3. Minor alteration, no change in sequence detected.
Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Table 2. P057-B3 probes arranged according to chromosomal location

Table 2a. *FANCD2*

Length (nt)	SALSA MLPA probe	FANCD2 exon[a]	Ligation site NM_001018115.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*71-73 (Exon 2)*		
265 #	01649-L01226	Exon 1	198 nt before exon 1	AGCTTCTCTTCA-CCGGGGCGCAGT	0.1 kb
330	01650-L19934	Exon 1	57 nt before exon 1	CTTCCGGCGCGG-AAGTTGGCGTCA	2.3 kb
172	02129-L07570	Exon 2	95-96	GAAGACTGTCAA-AATCTGAGGATA	5.8 kb
178	02130-L07571	Exon 4	298-299	ATAGCTTTCCAA-AAGAAGCTCTTT	0.7 kb
232	16148-L18317	Exon 6	487-486, reverse	TCAATCCCCAGA-AGCAGTTTGATG	4.6 kb
193	02131-L01624	Exon 9	702-703	GCAGCATGACAT-CATCACCAGCCT	1.9 kb
249	16149-L28607	Exon 10	794-793, reverse	GATTGGGACAGT-GAGTGAAGTATT	1.4 kb
214 # Δ	02132-L28606	Exon 12	988-989	GAGAAGTTGGAT-CTGCAGCATTGT	31.5 kb
256	16150-L18319	Exon 29	2844-2843, reverse	CCAGCTCTCGGA-AAAAAGCATGGG	3.6 kb
220	16147-L18316	Exon 30	3021-3022	GACACCTCCTAT-TGCCAGGAGAGT	3.3 kb
274	02137-L01630	Exon 32	3280-3281	CAGATTTTTCAT-GGGCTTTTTGCT	7.1 kb
283	02138-L01631	Exon 35	3588-3589	TGGGGATAAAGA-GAAGAGCAACAT	3.7 kb
364	16151-L18320	Exon 38	3856-3855, reverse	AGGAGTTTCTCT-TCATGAATCTGG	3.1 kb
301	02139-L19933	Exon 41	4062-4063	ACTGGAAACCTT-CCAGTTGGACAC	3.6 kb
310	02140-L01633	Exon 43a	12 nt after exon 43a	TATCTCTACAAA-ACCCACCAGAGT	
		stop codon	*4424-4426 (Exon 44)*		

Table 2b. *PALB2*

Length (nt)	SALSA MLPA probe	*PALB2* exon[a]	Ligation site NM_024675.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*154-156 (Exon 1)*		
142 »	07492-L09488	Exon 1	133-134	ACGGCTGCTCTT-TTCGTTCTGTCG	3.1 kb
427 »	07493-L28609	Exon 2	242-241, reverse	GGGCTAGTGTCT-TGCTGTATTCCC	0.2 kb
401	07494-L07156	Exon 3	341-340, reverse	GCTGCGGTGAGA-GATCCTGCTGAG	1.7 kb
355	07495-L28608	Exon 4	499-500	GCCCAGGAGGAT-TACCTATACAAA	6.1 kb
409	07496-L06744	Exon 5	2161-2162	CAGAAATGGAGG-ACTTAGAAGAGG	0.9 kb
373 »	07497-L07159	Exon 6	2694-2695	CTTCCTGCTTCT-GATAGCATAAAC	3.0 kb
208	16146-L18315	Exon 7	2 nt after exon 7	CTTCGCAGAGGT-AAGTGGGAATCT	2.1 kb
384	16152-L18321	Exon 8	1 nt before exon 8, reverse	AATACTGGAACC-TAAATAAAACAA	1.1 kb
156	07933-L32646	Exon 9	3096-3097	GTTAGTAGCAGT-GGGACCCTTTCT	1.6 kb
166 »	07501-L07163	Exon 10	3200-3201	TATACTAACTTT-TGCTGAGGTCCA	7.4 kb
292 »	07502-L07164	Exon 11	3321-3320, reverse	CAGACTGAAGCT-TGGTAAGAATCA	6.2 kb
161	16145-L18314	Exon 12	3482-3483	GCTGTACTGTCT-TCCTCCAGGGCA	4.3 kb
337 »	07504-L07166	Exon 13	3543-3544	TGTGCAGCAGCA-ATCTTGACTTCT	
		stop codon	*3712-3714 (Exon 13)*		

[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 may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.
» The ligation sites of these probes are the same, or in close proximity to probes present in SALSA MLPA P260 probemix.

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.

Related SALSA MLPA probemixes

P031 FANCA mix 1	Fanconi Anemia group A. Probes for the *FANCA* gene included.
P032 FANCA mix 2	Fanconi Anemia group A. Probes for the *FANCA* gene included.
P113 FANCB	Fanconi Anemia group B. Probes for the *FANCB* gene included.
P260 PALB2-RAD50-RAD51C-RAD51D	Probes for the *PALB2*, *RAD50*, *RAD51C* and *RAD51D* genes included.

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- Slater EP et al. (2010). PALB2 mutations in European familial pancreatic cancer families. *Clin Genet*. 78:490-4.
- 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.

Selected publications using SALSA MLPA Probemix P057 FANCD2-PALB2

- Adank MA et al. (2011). PALB2 analysis in BRCA2-like families. *Breast Cancer Res Treat*, 127(2), 357-362.
- Blanco A et al. (2012). Detection of a large rearrangement in PALB2 in Spanish breast cancer families with male breast cancer. *Breast Cancer Res Treat*. 132:307-15.
- Chang L et al. (2021). Novel diagnostic approaches for Fanconi anemia (FA) by single-cell sequencing and capillary nano-immunoassay. *Blood Science*, 3(1), 20-25.
- Francies FZ et al. (2018). Diagnosis of Fanconi Anaemia by ionising radiation-or mitomycin C-induced micronuclei. *DNA Repair (Amst)*. 61:17-24.
- Ghiorzo P et al. (2012). Contribution of germline mutations in the BRCA and PALB2 genes to pancreatic cancer in Italy. *Fam Cancer*. 11:41-7.
- Guenard F et al. (2010). Evaluation of the Contribution of the Three Breast Cancer Susceptibility Genes CHEK2, STK11, and PALB2 in Non-BRCA1/2 French Canadian Families with High Risk of Breast Cancer. *Genet Test Mol Biomarkers*. 14:515-26.
- Harinck F et al. (2012). Routine testing for PALB2 mutations in familial pancreatic cancer families and breast cancer families with pancreatic cancer is not indicated. *Eur J Hum Genet*. 20:577-9.

P057 product history	
Version	*Modification*
B3	Five reference probes have been replaced and several probes have a change in length but no change in the sequence targeted.
B2	Four reference probes have been replaced and one added.
B1	Probes for *FANCD2* exons 17, 23 and 28 has been removed (pseudo region). New probes added for *FANCD2* exons 10, 29, 30 and 38 and one probe (exon 6) redesigned. Three probes for *PALB2* have been redesigned. Six Reference probes have been replaced and one reference probe was added.
A2	Control fragments at 88-96-100-105 nt have been added.
A1	First release.

Implemented changes in the product description
Version B3-01 – 23 June 2021 (04P) - 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). - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. - Ligation sites of the probes targeting the *FANCD2* and *PALB2* genes updated according to new version of the NM_ reference sequence. *Version B2-01 – 10 October 2018 (01P)* - Product description restructured and adapted to a new template. - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. - A remark was added for probes with ligation sites similar or close to the probes in probemix P260. *Version 18 – 24 June 2015 (54)* - Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included). - Small textual changes on page 1 and 2. *Version 17 (48)* - Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added. *Version 16 (48)* - This product description has been changed to incorporate a new lot (lot number added, small changes in Table 1 and Table 2, new picture included). - Minor textual changes. *Version 15 (45)* - This product description has been changed to incorporate a new lot (lot number added, small changes in Table 1 and Table 2, new picture included). - Small textual changes page 1. Data analysis section has been modified. *Version 14 (43)* - This product description has been changed to incorporate a new lot (lot number added, small changes in Table 1 and Table 2, new picture included).

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