Product Description SALSA® MLPA® Probemix P454-A2 CGD

To be used with the MLPA General Protocol.

Version A2. As compared to version A1, three reference probes have been replaced and three reference probes have been removed. Five probes have been adjusted in probe length. For complete product history see page 7.

Catalogue numbers:
- **P454-025R:** SALSA MLPA Probemix P454 CGD, 25 reactions.
- **P454-050R:** SALSA MLPA Probemix P454 CGD, 50 reactions.
- **P454-100R:** SALSA MLPA Probemix P454 CGD, 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.mlpa.com).

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

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.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 P454 CGD is a **research use only (RUO)** assay for the detection of deletions or duplications in the *CYBA*, *CYBB*, *NCF2* and *NCF4* genes, which are associated with chronic granulomatous disease (CGD).

Patients with chronic granulomatous disease (CGD) suffer from recurrent, life-threatening bacterial and fungal infections of skin, airways, lymph nodes, liver, brain and/or bones. CGD is a rare (~1:250 000 births) recessive disorder caused by mutations in any one of the five components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in phagocytes. This enzyme generates superoxide and is essential for intracellular killing of pathogens by phagocytes. The genes encoding the five NADPH oxidase components are *CYBB* (located on the X-chromosome), and the autosomal genes *CYBA*, *NCF1*, *NCF2* and *NCF4*. Approximately 70% of the CGD patients have a mutation in *CYBB*. The remainder of the patients have mutations in *NCF1* (~ 20%), in *CYBA* (~ 5%) or in *NCF2* (~ 5%). Only one patient with *NCF4* mutations has been reported (Matute et al. 2009). Copy number variations account for approximately 15% of pathogenic variants in each of the *CYBA*, *CYBB* and *NCF2* genes.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK99496/.

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 *CYBA* exon numbering used in this P454-A2 CGD product description is the exon numbering from the RefSeq transcript NM_000101.4, which is identical to the LRG_52 sequence. The *CYBB* exon numbering is the exon numbering from the RefSeq transcript NM_000397.4, which is identical to the LRG_53 sequence. The *NCF2* exon numbering is the exon numbering from the RefSeq transcript NM_000433.3, which is identical to the LRG_88 sequence. The *NCF4* exon numbering is the exon numbering from the RefSeq

transcript NM_013416.3, which is identical to the LRG_159 sequence. The exon numbering and NM_ sequences used have been retrieved on 06/2020. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Probemix content: The SALSA MLPA Probemix P454-A2 CGD contains 38 MLPA probes with amplification products between 129 and 454 nucleotides (nt). This includes three probes for the *CYBA* gene, 12 probes for the *CYBB* gene, ten probes for the *NCF2* gene and four probes for the *NCF4* gene and. Due to the presence of pseudogenes of *NCF1*, it was not possible to include probes specific for the *NCF1* gene in this probemix. In addition, nine 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.mlpa.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.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal of 88 nt and 96 nt fragment 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.mlpa.com).

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

Required specimens: Extracted DNA, 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 unrelated individuals who are from families without a history of CGD. 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 MLPA General Protocol.

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/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample 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.mlpa.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 dosage quotient (DQ) 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 DQ of the probes can be used to interpret MLPA results when **reference samples of the same sex** have been used:

Copy number status		Dosage quotient
Autosomal sequences and X chromosome sequences in females	**X chromosome sequences in males**	
Normal	Normal	0.80 < DQ < 1.20
Homozygous deletion	Deletion	DQ = 0
Heterozygous deletion		0.40 < DQ < 0.65
Heterozygous duplication		1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	Duplication	1.75 < DQ < 2.15
Ambiguous copy number		All other values

- 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. Incomplete DNA denaturation (e.g. due to salt contamination) can 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.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. 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: lower injection voltage / injection time settings, 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 *CYBA*, *CYBB*, *NCF2* and *NCF4* genes are small (point) mutations, which will not be detected by using SALSA MLPA Probemix P454 CGD.
- 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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.

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

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

Mutation databases:
CYBA: https://databases.lovd.nl/shared/genes/CYBA
CYBB: https://databases.lovd.nl/shared/genes/CYBB
NCF2: https://databases.lovd.nl/shared/genes/NCF2
NCF4: https://databases.lovd.nl/shared/genes/NCF4
We strongly encourage users to deposit positive results in the Leiden Open Variation Database (LOVD). 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 SNPs and unusual results (e.g., a duplication of *CYBB* exons 3 and 5 but not exon 4) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P454-A2 CGD

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]				
		Reference	*CYBB*	*NCF2*	*CYBA*	*NCF4*
64-105	Control fragments – see table in probemix content section for more information					
129	Reference probe 18709-L26847	5q31				
136	**CYBB probe** 19770-L26576		**Exon 4**			
142	**NCF2 probe** 19771-L26577			**Exon 1**		
148	**NCF4 probe** 19772-L26578					**Exon 7**
167	**NCF2 probe** 19773-L28156			**Exon 12**		
174	**CYBB probe** 19774-L28155		**Exon 13**			
178 *	Reference probe 08599-L28166	17p11				
184	**CYBB probe** 19775-L26581		**Exon 2**			
190	**NCF2 probe** 19776-L26582			**Exon 4**		
196	**CYBB probe** 19777-L28157		**Exon 12**			
202	**CYBB probe** 19778-L26584		**Exon 8**			
208	**CYBB probe** 19779-L27741		**Exon 1**			
221	**NCF2 probe** 19781-L28158			**Exon 8**		
229	**CYBB probe** 19782-L26588		**Exon 10**			
234 ¥	**NCF2 probe** 19783-L27745			**Exon 2**		
241	**NCF2 probe** 19784-L27744			**Exon 15**		
247 Ж	**NCF4 probe** 19785-SP0865-L29114					**Exon 1**
256 ¥	**CYBB probe** 22816-L27742		**Exon 6**			
265	**CYBA probe** 19786-L26592				**Exon 5**	
268 *	Reference probe 09064-L27989	19p13				
283 ¥	**CYBA probe** 22817-L26600				**Exon 3**	
292	**CYBB probe** 19787-L26593		**Exon 9**			
300 ¥ Ж	**CYBA probe** 22818-SP0866-L26605				**Exon 1**	
310 ¥	**NCF4 probe** 22819-L26604					**Exon 4**
323 *	Reference probe 20640-L26608	6q12				
337	Reference probe 10376-L10928	9q34				
346	**CYBB probe** 19788-L26594		**Exon 5**			
355	**NCF2 probe** 19789-L26595			**Exon 6**		
363	**NCF4 probe** 19790-L26596					**Exon 9**
373	Reference probe 08602-L25186	10q26				
379	Reference probe 12558-L18075	11p13				
393	**NCF2 probe** 19791-L26597			**Exon 14**		
400	Reference probe 17960-L22873	18q21				
408	**CYBB probe** 19792-L26598		**Exon 7**			
418	**NCF2 probe** 19793-L26599			**Exon 7**		
436	**CYBB probe** 19795-L26601		**Exon 3**			
445	**NCF2 probe** 19796-L26602			**Exon 9**		
454	Reference probe 05916-L23023	21q11				

a) See above section on exon numbering for more information.

* New in version A2.
¥ Changed in version A2. Minor alteration, no change in sequence detected.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

Table 2. P454-A2 probes arranged according to chromosomal location

Table 2a. *NCF2* gene

Length (nt)	SALSA MLPA probe	*NCF2* exon[a]	Ligation site NM_000433.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*276-278 (Exon 1)*		
142	19771-L26577	Exon 1	95-96	GTGGGGACATTT-CCTGATGCATTT	3.5 kb
234 ¥	19783-L27745	Exon 2	8 nt before exon 2 *reverse*	AGGCCTGAGGAG-AGAAGGGTCCAG	12.5 kb
190	19776-L26582	Exon 4	726-727	CCACGAGCATGA-AGTCTGAGCCCA	3.7 kb
355	19789-L26595	Exon 6	894-895	AGGTCGTGGCAT-CTGTGGTGGATC	1.6 kb
418	19793-L26599	Exon 7	27 nt before exon 7	TTGCCTTTTCCG-TTTTCACTTCTC	1.9 kb
221	19781-L28158	Exon 8	1026-1027	TGCTATTTGGGT-TTGTGCCTGAGA	0.3 kb
445	19796-L26602	Exon 9	intron-1131	GCTGCACTGCAG-AAGGGGCTTGTT	3.5 kb
167	19773-L28156	Exon 12	1400-1401	TACAGCCAGGTC-CGGGACATGGTG	3.3 kb
391	19791-L26597	Exon 14	1675-1676	GGCACTCTTCAG-TTATGAGGCTAC	4.1 kb
241	19784-L27744	Exon 15	1894-1895	GAAAATGAAGCC-CTATTACTTGTT	
		stop codon	*1854-1856 (Exon 15)*		

Table 2b. *CYBA* gene

Length (nt)	SALSA MLPA probe	*CYBA* exon[a]	Ligation site NM_000101.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*41-43 (Exon 1)*		
300 ¥ Ж	22818-SP0866-L26605	Exon 1	2 nt after exon 1; 32 nt after exon 1	TCCGGCCTGAGT-30nt spanning oligo-GCCTGGAGGGGT	3.7 kb
283 ¥	22817-L26600	Exon 3	6 nt before exon 3	TTAACCACATGG-CTTCAGTGTGGC	1.0 kb
265	19786-L26592	Exon 5	356-357	GCTTCCTGCTGG-CCACCATCCTTG	
		stop codon	*626-628 (Exon 6)*		

Table 2c. *NCF4* gene

Length (nt)	SALSA MLPA probe	*NCF4* exon[a]	Ligation site NM_013416.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*185-187 (Exon 1)*		
247 Ж	19785-SP0865-L29114	Exon 1	176-177; 205-206	CCCTGGGACCAT-29 nt spanning oligo-CGGGCCGAGAGG	6.3 kb
310 ¥	22819-L26604	Exon 4	519-520	CCTCAACGCCTA-CATGAAGGTACC	4.9 kb
148	19772-L26578	Exon 7	745-746	AACAGCAAACTG-GAGCTGAATTTC	5.5 kb
363	19790-L26596	Exon 9	1477-1478	GAGCAGTGAGGG-GACACCAGCAAA	
		stop codon	*1229-1231 (Exon 8)*		

Table 2d. *CYBB* gene

Length (nt)	SALSA MLPA probe	*CYBB* exon[a]	Ligation site NM_000397.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*20-22 (Exon 1)*		
208	19779-L27741	Exon 1	7-8	ACAACACATTCA-ACCTCTGCCACC	2.1 kb
184	19775-L26581	Exon 2	148-149	TTTTACACAAGA-AAACTTCTTGGG	1.4 kb
436	19795-L26601	Exon 3	5 nt after exon 3	CAGTGCGGTAAG-AGAAAATGTTTT	8.4 kb
136	19770-L26576	Exon 4	275-276	ATCCAAAGTGCT-GCTCAACAAGAG	1.8 kb
346	19788-L26594	Exon 5	27 nt after exon 5	TCTGACTTAGAT-ATTCTCTAGGCC	2.2 kb
256 ¥	22816-L27742	Exon 6	537-538	GGCTGTGACCCT-GTTGGCAGGCAT	3.1 kb
408	19792-L26598	Exon 7	796-797	GAATGCCCAATC-CCTCAGTTTGCT	2.2 kb
202	19778-L26584	Exon 8	861-862	GTTTCTGTATCT-CTGTGAGAGGTT	2.8 kb
292	19787-L26593	Exon 9	1138-1139	GGCTGTGATAAG-CAGGAGTTTCAA	1.0 kb
229	19782-L26588	Exon 10	1308-1309	ATATTGCAATAA-CGCCACCAATCT	4.6 kb
196	19777-L28157	Exon 12	17 nt after exon 12	GAGTCTGTCACC-AAGATGTTTTTG	1.8 kb
174	19774-L28155	Exon 13	2338-2339	TACTAATACAGC-AGGGTAACTGGG	
		stop codon	*1730-1732 (Exon 20)*		

a) See above section on exon numbering for more information.

b) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

¥ Changed in version A2. Minor alteration, no change in sequence detected.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

References

- Matute JD et al. (2009). A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40 phox and selective defects in neutrophil NADPH oxidase activity. *Blood*. 114:3309-15.
- 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.
- 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 P454 CGD

- Teimourian S et al. (2018). Characterization of 4 new mutations in the CYBB gene in 10 Iranian families with X-linked chronic granulomatous disease. *J Pediatr Hematol Oncol*. 40:e268-72.

P454 Product history	
Version	*Modification*
A2	Three reference probes have been replaced and three reference probes have been removed. Five probes have been adjusted in probe length.
A1	First release.

Implemented changes in the product description
Version A2-01 — 10 July 2020 (02P) - 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). - Ligation sites of the probes targeting the genes *CYBA*, *CYBB*, *NCF2* and *NCF4* updated according to new version of the NM_ reference sequence.

More information: www.mlpa.com; www.mlpa.eu	
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