Product Description
SALSA® MLPA® Probemix P494-A1 NBN

To be used with the MLPA General Protocol.

Version A1
For complete product history see page 7.

Catalogue numbers:
- **P494-025R:** SALSA MLPA Probemix P494 NBN, 25 reactions.
- **P494-050R:** SALSA MLPA Probemix P494 NBN, 50 reactions.
- **P494-100R:** SALSA MLPA Probemix P494 NBN, 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 P494 NBN is a **research use only (RUO)** assay for the detection of deletions or duplications in the *NBN* gene, which is associated with Nijmegen breakage syndrome.

Nijmegen breakage syndrome (NBS) is characterised by progressive microcephaly, intrauterine growth retardation and short stature, recurrent sinopulmonary infections, an increased risk of cancer, and premature ovarian failure in females. It is inherited in an autosomal recessive manner, but carriers have an increased risk of breast cancer (Bogdanova et al. 2008). The prevalence is unknown, but it is most common in the Central and Eastern European populations. The disease is caused by mutations in the *NBN* gene (previously known as *NBS1*). The *NBN* gene encodes nibrin, which is part of a double-strand break repair protein complex involved in cell-cycle checkpoint regulation (Varon et al. 1998).

The *NBN* gene (16 exons) spans ~51 kb of genomic DNA and is located on chromosome 8q21.3, ~91 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1176/.

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 *NBN* exon numbering used in this P494-A1 NBN product description is the exon numbering from the LRG_158 sequence. The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. As changes to the

databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date.

Probemix content

The SALSA MLPA Probemix P494-A1 NBN contains 27 MLPA probes with amplification products between 172 and 373 nucleotides (nt). This includes 18 probes for the *NBN* gene (one probe for each exon, two probes for exon 16 and one upstream probe). 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.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 Nijmegen breakage syndrome. 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 NA02030 and NA03134 from the Coriell Institute have been tested with this P494-A1 probemix at MRC Holland and can be used as positive control samples to detect a heterozygous duplication and a heterozygous deletion of the *NBN* gene, respectively. 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.

P494 specific note:

- Please note that a novel *NBN* processed pseudogene might be present in a small part of the population (< 1:1000 individuals) (Mancini et al. 2015). The presence of this pseudogene might result in an apparent duplication of many *NBN* probes. This new pseudogene is not present in the human reference genome and is probably clinically insignificant.

Limitations of the procedure

- The most reported genetic defect in the *NBN* gene is the c.657_661del5 mutation, which will not be detected by using SALSA MLPA Probemix P494 NBN. Other small (point) mutations will also not be detected by this probemix.
- 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

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.

***NBN* mutation database**

https://databases.lovd.nl/shared/genes/NBN. We strongly encourage users to deposit positive results in the Leiden Open Variation Database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNVs and unusual results (e.g., a duplication of *NBN* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P494-A1 NBN

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*NBN*
64-105	Control fragments – see table in probemix content section for more information		
172	Reference probe 10922-L25079	9q	
178	Reference probe 04567-L03956	16q	
186 Ø	**NBN probe** 23058-L32550		upstream
190	**NBN probe** 23059-L32551		**Exon 5**
196	**NBN probe** 23060-L32552		**Exon 16**
202	Reference probe 10880-L14426	15q	
208	**NBN probe** 23061-L32553		**Exon 9**
214	**NBN probe** 23062-L32554		**Exon 13**
220	**NBN probe** 23063-L32555		**Exon 3**
226	Reference probe 20173-L32722	2p	
233	**NBN probe** 23064-L32723		**Exon 12**
238	**NBN probe** 23065-L32557		**Exon 7**
247	**NBN probe** 23066-L32558		**Exon 4**
255	Reference probe 06236-L27147	21q	
264	**NBN probe** 23067-L32559		**Exon 1**
274	**NBN probe** 23068-L32560		**Exon 15**
283	**NBN probe** 23069-L32561		**Exon 6**
292	Reference probe 15724-L17704	12q	
301	**NBN probe** 23070-L32562		**Exon 16**
310	**NBN probe** 23071-L32563		**Exon 10**
319	**NBN probe** 23072-L32564		**Exon 8**
331	Reference probe 08905-L24614	11p	
337	**NBN probe** 23073-L32565		**Exon 2**
346	**NBN probe** 23074-L32566		**Exon 14**
355	**NBN probe** 23075-L32567		**Exon 11**
366	Reference probe 14768-L29121	1q	
373	Reference probe 21994-L30832	4p	

[a] See section Exon numbering on page 1 for more information.

Ø Upstream probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

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. *NBN* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*NBN* exon[a]	Ligation site NM_002485.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*107-109 (Exon 1)*		
186 Ø	23058-L32550	upstream	1694 nt before exon 1	ATAGTTTGATCG-ATGTATTACCAA	
264	23067-L32559	Exon 1	48-49	GTATCCGCGCTC-GTCTAGCAGCCC	1.7 kb
337	23073-L32565	Exon 2	180-181	CGTTGAGTACGT-TGTTGGAAGGAA	1.8 kb
220	23063-L32555	Exon 3	314-313 *reverse*	CTTAGAATTATC-TTTTAATGTCAA	1.3 kb
247	23066-L32558	Exon 4	453-452 *reverse*	AACAAGAAGAGC-ATGCAACCAAAG	0.6 kb
190	23059-L32551	Exon 5	625-626	CCAATTGTAAAG-CCAGAATATTTT	2.6 kb
283	23069-L32561	Exon 6	714-715	TCTTGATGAACC-ATCTATTGGAAG	7.0 kb
238	23065-L32557	Exon 7	966-967	TGACTGTCAGAA-GAAATGGATTCA	0.9 kb
319	23072-L32564	Exon 8	1069-1068 *reverse*	GGATCACAGTAA-TTCTTTGTAGTC	6.0 kb
208	23061-L32553	Exon 9	1180-1181	AGCGCCCCAGTG-AACACTACAACA	5.7 kb
310	23071-L32563	Exon 10	1338-1337 *reverse*	TATTATTATTAG-AGCTTGTTTTGC	3.3 kb
355	23075-L32567	Exon 11	1705-1706	TCTATTGTGAAA-AATTCTGCCAGT	1.9 kb
233	23064-L32723	Exon 12	2004-2003 *reverse*	TTTCTTTAGCTG-ACCATAGTGAGT	5.7 kb
214	23062-L32554	Exon 13	2074-2073 *reverse*	CTAAATTCAGTC-AATAACAGCTTT	1.6 kb
346	23074-L32566	Exon 14	2219-2220	ACATCATTGGAG-GATCAGATCTAA	2.9 kb
274	23068-L32560	Exon 15	10 nt after exon 15 *reverse*	AGAAAGGTGAAT-CAAACTTTACCT	6.3 kb
301	23070-L32562	Exon 16	2420-2419 *reverse*	TTGGCCTGAAGT-AGATGCTTACTA	1.5 kb
196	23060-L32552	Exon 16	3780-3779 *reverse*	CTTGGTAATTAT-GATACATGGCTA	1.4 kb
		stop codon	*2369-2371 (Exon 16)*		

[a] See section Exon numbering on page 1 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.

Ø Upstream probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

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.

References

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P494 product history	
Version	*Modification*
A1	First release.

Implemented changes in the product description
Version A1-01 – 28 July 2021 (04P) - Not applicable, new document.

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