Product Description
SALSA® MLPA® Probemix P078-D2 Breast tumour

To be used with the MLPA General Protocol.

Version D2
For complete product history see page 11.

Catalogue numbers:
- **P078-025R:** SALSA MLPA Probemix P078 Breast tumour, 25 reactions.
- **P078-050R:** SALSA MLPA Probemix P078 Breast tumour, 50 reactions.
- **P078-100R:** SALSA MLPA Probemix P078 Breast tumour, 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 P078 Breast tumour is a **research use only (RUO)** assay for the detection of deletions or duplications in 6q25 *(ESR1)*, 7p11 *(EGFR)*, 8p11-p12 *(ZNF703, FGFR1, ADAM9, IKBKB)*, 8q13-q24 *(PRDM14, MTDH, MYC)*, 11q13 *(CCND1, EMSY)*, 16q22 *(CDH1)*, 17q11-q25 *(CPD, MED1, ERBB2, CDC6, TOP2A, MAPT, PPM1D, BIRC5)*, 19q12 *(CCNE1)* and 20q13 *(AURKA)*, several of which are suggested to be of diagnostic/clinical importance in breast cancer. See Table 1 and Table 2 for more detailed information about all included chromosomal regions and genes.

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
Matched Annotation from NCBI and EMBL-EBI (MANE): https://www.ncbi.nlm.nih.gov/refseq/MANE/ and http://tark.ensembl.org/
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 exon numbering used in this P078-D2 Breast tumour product description is the exon numbering from the MANE transcripts, as indicated in Table 2. The *EMSY*, *ERBB2, BIRC5* and *AURKA* exon numbering has been changed according to MANE select; the exon numbering used in previous versions of this product description (NM_020193.4, LRG_724, NM_001012271.1 and NM_198433.2, respectively) can be found in between brackets in Table 2. **From description version D2-02 onwards, we have adopted the MANE exon numbering. Please be aware that the MANE and previous exon numbering do not always correspond, and MANE exon numbering used here may differ from literature.** 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 P078-D2 Breast tumour contains 55 MLPA probes with amplification products between 127 and 500 nt. This includes in total 41 probes for the following chromosomal regions: 6q25 *(ESR1)*, 7p11 *(EGFR)*, 8p11-p12 *(ZNF703, FGFR1, ADAM9, IKBKB)*, 8q13-q24 *(PRDM14, MTDH, MYC)*, 11q13 *(CCND1, EMSY)*, 16q22 *(CDH1)*, 17q11-q25 *(CPD, MED1, ERBB2, CDC6, TOP2A, MAPT, PPM1D, BIRC5)*, 19q12 *(CCNE1)* and 20q13 *(AURKA)*. In addition, 14 reference probes are included which target relatively copy number stable regions in various tumour types, including breast cancer. The identity of the genes detected by the reference probes is available in Table 3. Complete probe sequences 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). More information on the use of MLPA in tumour applications can be found in Hömig-Hölzel and Savola (2012).

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, which includes DNA derived from paraffin-embedded tissues, 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. More information on the use of FFPE tissue samples for MLPA can be found in Atanesyan et al. (2017).

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 healthy individuals without a history of breast tumour. 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 NA07994, NA07081, NA02030, NA14485, NA03999, NA00959, NA12074, NA16445 and NA08123 from the Coriell Institute, and HEP-G2 and 8-MG-BA from Leibniz Institute DSMZ have been tested with this P078-D2 probemix at MRC Holland and can be used as a positive control samples to detect copy number alterations, described in the table below. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Chromosomal position of copy number alteration (hg18)*	Altered target genes in P078-D2	Expected copy number alteration
NA07994	Coriell Institute	6q25.1	*ESR1*	Heterozygous duplication
NA07081	Coriell Institute	7p11.2	*EGFR*	Heterozygous duplication
NA14485	Coriell Institute	8p11.21-p12	*ZNF703, FGFR1, ADAM9, IKBKB*	Heterozygous duplication
NA02030	Coriell Institute	8p11.21-q24.1	*ZNF703, FGFR1, ADAM9, IKBKB, PRDM14, MTDH, MYC*	Heterozygous duplication
NA03999	Coriell Institute	8q24.21	*MYC*	Heterozygous deletion
NA00959	Coriell Institute	11q13.2-q13.5	*CCND1, EMSY*	Heterozygous duplication
NA12074	Coriell Institute	16q22.1	*CDH1*	Heterozygous deletion
NA16445	Coriell Institute	17q25.3	*BIRC5*	Heterozygous duplication
NA08123	Coriell Institute	20q13.2	*AURKA*	Heterozygous duplication
HEP-G2◊	DSMZ	6q25.1	*ESR1*	Gain
		16q22.1	*CDH1*	Gain
		17q12-q25.3	*MED1, ERBB2, CDC6, TOP2A, MAPT, PPM1D, BIRC5*	Gain
		20q13.2	*AURKA*	Gain
8-MG-BA◊	DSMZ	7p11.2	*EGFR*	Gain
		8q13.3-q24.21	*PRDM14, MTDH, MYC*	Gain
		11q13.2-q13.5	*CCND1, EMSY*	Gain
		17q21.31-q25.3	*MAPT, PPM1D, BIRC5*	Gain
		19q12	*CCNE1*	Gain
		20q13.2	*AURKA*	Gain

* 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 P078-D2 Breast tumour probemix.

◊ In this indicated cell line sample some of the reference probes are also affected by CNAs.

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. When this criterion 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.

Please note that these above mentioned final ratios are only valid for germline testing. Final ratios are affected both by percentage of tumour cells and by possible subclonality.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in subclonal cases.
- 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 or in or near the *CCNE* and *BIRC5* genes. 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.

P078 specific note

- In samples from tumour tissues, reference probes are more prone to have deviating copy number results as compared to blood derived germline samples. When regions targeted by reference probes are affected by copy number alterations, it can help to turn the slope correction off in Coffalyser.Net analysis to get the correct copy number interpretation on the target region.

Limitations of the procedure

- In most populations, the major cause of genetic defects in chromosomal regions and genes included in this probemix are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P078 Breast tumour.
- 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.
- MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Gains or losses of genomic regions or genes may not be detected if the percentage of tumour cells is low. In addition, subclonality of the aberration affects the final ratio of the corresponding probe. Furthermore, there is always a possibility that one or more reference probes *do* show a copy number alteration in a patient sample, especially in breast cancer samples with more chaotic karyotypes.

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.

COSMIC mutation database

http://cancer.sanger.ac.uk/cosmic. We strongly encourage users to deposit positive results in the COSMIC database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report false positive results due to SNVs and unusual results (e.g. an amplification of *ERBB2* exons 3 and 15, but not exon 9) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P078-D2 Breast tumour

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		Location (hg18) in kb
		Reference	Target region	
64-105	Control fragments – see table in probemix content section for more information			
127 *	Reference probe 15370-L19110	7q11		07-075,448
133 ∫	**EMSY probe** 09173-L21898		11q13.5	11-075,902
139	**ZNF703 probe** 17592-L28959		8p12	08-037,673
143	Reference probe 14199-L15813	2q13		02-108,894
148	**ERBB2 probe** 00675-L18842		17q12	17-035,118
154	**IKBKB probe** 11993-L22094		8p11.21	08-042,293
158	**MYC probe** 20780-L28945		8q24.21	08-128,822
166 «	**CCNE1 probe** 02881-L02348		19q12	19-035,005
172	**TOP2A probe** 11994-L12822		17q21.2	17-035,818
177 ±	**CDH1 probe** 16884-L21554		16q22.1	16-067,405
184	Reference probe 10710-L11292	6p12		06-052,016
190 «	**CCNE1 probe** 09170-L09344		19q12	19-035,000
196	**CDC6 probe** 08611-L13204		17q21.2	17-035,699
202	**ERBB2 probe** 17591-L12908		17q12	17-035,122
208 ¥	**PPM1D probe** 22339-L21557		17q23.2	17-056,056
214	**ESR1 probe** 11996-L12824		6q25.1	06-152,424
220 *	Reference probe 13789-L31486	10q23		10-095,547
226	**CPD probe** 09628-L21977		17q11.2	17-025,795
232	**ADAM9 probe** 11997-L21978		8p11.23	08-038,994
238	**AURKA probe** 17365-L21549		20q13.2	20-054,392
244	**ESR1 probe** 11998-L21550		6q25.1	06-152,457
250	**ERBB2 probe** 12048-L21551		17q12	17-035,136
256 ∫	**EMSY probe** 09175-L09349		11q13.5	11-075,927
262	**ZNF703 probe** 17595-L21581		8p12	08-037,675
268 ±	**EGFR probe** 05969-L20430		7p11.2	07-055,234
274	Reference probe 13796-L15290	3q25		03-157,716
280 ¥	**MYC probe** 14870-L26915		8q24.21	08-128,818
285 #	**MTDH probe** 04151-L21553		8q22.1	08-098,742
292	**CCND1 probe** 00583-L00148		11q13.2	11-069,175
298 *	Reference probe 18378-L31470	12p11		12-032,840
310	Reference probe 09065-L09234	19p13		19-013,289
316	**ERBB2** probe 00986-L28769		17q12	17-035,127
323	**BIRC5** probe 03717-L28768		17q25.3	17-073,722
330	**TOP2A** probe 11999-L21541		17q21.2	17-035,813
337	**MTDH** probe 04152-L21907		8q22.1	08-098,788
346 *	Reference probe 03580-L02941	3p22		03-038,573
352	**MED1** probe 09963-L21558		17q12	17-034,841
358	**CDH1** probe 15622-L21559		16q22.1	16-067,329
365	**TOP2A** probe 12000-L28949		17q21.2	17-035,817
373 ¥	Reference probe 05953-L30687	2p22		02-032,222
380	**FGFR1** probe 01046-L28764		8p12	08-038,434
385	Reference probe 09717-L28947	12q24		12-116,200
392 ± «	**BIRC5** probe 03025-L28946		17q25.3	17-073,724
400	**FGFR1** probe 04440-L03826		8p12	08-038,392
409	Reference probe 01237-L27145	10p14		10-012,019
418	**MAPT** probe 20778-L28948		17q21.31	17-041,423
427	**EGFR** probe 02063-L03283		7p11.2	07-055,191
436 «	**BIRC5** probe 03189-L02540		17q25.3	17-073,722
445	**PRDM14** probe 12002-L12830		8q13.3	08-071,130

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		Location (hg18) in kb
		Reference	Target region	
452	Reference probe 12459-L13460	14q24		14-076,832
463	**IKBKB** probe 12003-L21560		8p11.21	08-042,303
472	**CCND1** probe 05402-L21561		11q13.2	11-069,168
484	**AURKA** probe 17590-L21028		20q13.2	20-054,382
494	Reference probe 20779-L21727	4q22		04-090,869
500	Reference probe 06676-L21510	11p15		11-006,369

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

* New in version D2 (from lot D2-1018 onwards).

¥ Changed in version D2. Minor alteration, no change in sequence detected.

± SNPs rs187862045, rs767507216 and rs371975672 could influence probe signals at 177 nt, 268 nt and 392 nt, respectively. 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.

∫ Name of *C11ORF30* gene has been updated to *EMSY* according to the HUGO nomenclature throughout the document.

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 tables above. Single probe aberration(s) must be confirmed by another method.

Table 2. P078-D2 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene/Exon[a]	Location / Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
ESR1 gene, 6q25.1. Gain or amplification of the estrogen receptor alpha *(ESR1)* gene is a relatively frequent event in breast cancer and suggested to be a clinical marker for response to hormone therapy (Albertson et al. 2012; Ooi et al. 2012). It is important to note that with FISH, up to 20% of breast cancer cases have been reported to have an amplified *ESR1* gene, but a study showed that FISH is also detecting accumulated *ESR1* transcripts leading to false positive results. MLPA, detecting copy number changes at the DNA level, would therefore provide more accurate results on *ESR1* copy number analysis than FISH (Ooi et al. 2012).					
214	11996-L12824	***ESR1***, ex 6	NM_000125.4; 1515-1516	TTCGACATGCTG-CTGGCTACATCA	33.4 kb
244	11998-L21550	***ESR1***, ex 7	NM_000125.4; 1628-1629	GTCCAGCACCCT-GAAGTCTCTGGA	-
EGFR gene, 7p11.2. *EGFR* amplification is detected in 8% of breast cancers and high expression levels of the *EGFR* gene are suggested to be an adverse prognostic factor for survival (Park et al. 2007). However, both the frequency and the prognostic value of *EGFR* amplification in breast cancer is still controversial and under debate (Ali et al. 2017).					
427	02063-L03283	***EGFR***, ex 8	NM_005228.5; 1215-1216	AGCTATGAGATG-GAGGAAGACGGC	42.9 kb
268 ±	05969-L20430	***EGFR***, ex 23	NM_005228.5; 3037-3038	AGATCTCCTCCA-TCCTGGAGAAAG	20.2 **M**b To *POR* ref. gene
8p11-p12 amplifications Amplification of 8p11-p12 is detected in ~15% of breast cancer patients and it is associated with poor prognosis (Yang et al. 2010). ***ZNF703*** gene amplification at 8p12 and overexpression of this gene has been shown to have a strong impact on the pathogenesis of luminal B breast cancers (Holland et al. 2011; Sircoulomb et al. 2011). ***FGFR1*** gene amplification at 8p12 is suggested to be the best marker of poor prognosis in this chromosomal area. Moreover, *FGFR1* is a putative therapeutic target, as it is a major contributor in endocrine therapy resistance (Turner et al. 2010). ***IKBKB*** gene at 8p11.21, codes for a kinase associated with IKK/NF-kB activation pathway, which makes it a potential therapeutic target within the 8p11-12 amplicon (Chin et al. 2006).					
139	17592-L28959	***ZNF703***, ex 1	NM_025069.3; 207-208	CAAATGAGCGAT-TCGCCCGCTGGA	2.5 kb
262	17595-L21581	***ZNF703***, ex 2	NM_025069.3; 1823-1824	CACTTTGGGCCT-AAGCCGGTACCA	716.4 kb
400	04440-L03826	***FGFR1***, ex 14	NM_023110.3; 2609-2610	TGCATACACCGA-GACCTGGCAGCC	42.6 kb
380	01046-L28764	***FGFR1***, ex 2	NM_023110.3; 732-733	CAACCTCTAACT-GCAGAACTGGGA	559.8 kb
232	11997-L21978	***ADAM9***	8p11.23	TGAGCACATCAT-TTATCGAATGGA	3.3 **M**b

Length (nt)	SALSA MLPA probe	Gene/Exon[a]	Location / Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
154	11993-L22094	***IKBKB***, ex 10	NM_001556.3; 1029-1030	CAACTGATGCTG-ATGTGGCACCCC	9.8 kb
463	12003-L21560	***IKBKB***, ex 20	NM_001556.3; 2229-2230	GCCTCTCGACTT-AGCCAGCCTGGG	28.8 **M**b
8q amplifications ***MTDH*** gene activation by 8q22.1 genomic gain promotes chemoresistance and metastasis of breast cancer (Hu et al. 2009; Tokunaga et al. 2014). ***MYC*** gene amplification at 8q24.21 is detected in ~15% of breast cancer patients and is a marker of poor survival (Deming et al. 2000).					
445	12002-L12830	***PRDM14***	8q13.3	CACTCTGGAGAC-AGACCATACCAG	27.6 **M**b
285 #	04151-L21553	***MTDH***, ex 2	NM_178812.4; 763-764	ACCTCAAAGTGT-AACAGCAAAGCA	45.6 kb
337	04152-L21907	***MTDH***, ex 8	NM_178812.4; 1553-1554	GAAGAAAGAGCT-TCACTTCTAAAG	30.0 **M**b
280	14870-L26915	***MYC***, ex 1	NM_002467.6; 242-243	CTGGAACTTACA-ACACCCGAGCAA	4.3 kb
158	20780-L28945	***MYC***, ex 3	NM_002467.6; 1520-1521	GAACGAGCTAAA-ACGGAGCTTTTT	-
11q13 amplifications ***CCND1*** gene amplifications, at 11q13.2, are detected in ~15% of breast cancer patients and are associated with poor overall survival in ER+ patients (Holm et al. 2012). High expression levels of *CCND1* were shown to associate with poor response to trastuzumab treatment in ER+ patients (Tanioka et al. 2014). ***EMSY*** (previously known as *C11ORF30*) gene amplifications, at 11q13.5, are detected in 7-13% of breast cancers and are suggested to associate with poor clinical outcome (Kirkegaard et al. 2008).					
472	05402-L21561	***CCND1***, ex 3	NM_053056.3; 598-599	CCTGGTGAACAA-GCTCAAGTGGAA	7.3 kb
292	00583-L00148	***CCND1***, ex 5	NM_053056.3; 927-928	CCCTGCTGGAGT-CAAGCCTGCGCC	6.7 **M**b
133 ∫	09173-L21898	***EMSY***, ex 11 (10)	NM_001300942.2; 1597-1598	AACCAAGTAAAA-TCTTACCCAAAC	24.5 kb
256 ∫	09175-L09349	***EMSY***, ex 17 (16)	NM_001300942.2; 2637-2638	ATGACCCAGGAA-AAGAGACATTCT	-
CDH1 gene, 16q22.1. Loss of heterozygosity and deletions affecting the 16q arm are one of the most common genetic alterations in breast cancer, occurring in ~50% of all ductal carcinomas and even more frequently in lobular breast cancer. Loss of E-cadherin *(CDH1)* is thought to contribute to progression in breast cancer, especially in ductal and lobular breast carcinomas (Chalmers et al. 2001).					
358	15622-L21559	***CDH1***, ex 1	NM_004360.5; 44-45	TTGCGGAAGTCA-GTTCAGACTCCA	76.1 kb
177 ±	16884-L21554	***CDH1***, ex 9	NM_004360.5; 1422-1423	AGTGAACAACGA-TGGCATTTTGAA	-
17q amplifications ***ERBB2*** (HER2-Neu) gene at 17q12, is amplified in 15-30% of breast cancers. Amplification of *ERBB2* defines an aggressive subtype of breast cancer that can be treated with targeted therapy (Trastuzumab/Herceptin). Moreover, amplification of *ERBB2* has been shown to correlate with poor prognosis and resistance to conventional adjuvant chemotherapy and tamoxifen (Slamon et al. 1987) and is the most important predictive factor of response to HER2-targeted therapies (Singh et al. 2014). ***CPD*** and ***MED1*** genes centromeric and ***CDC6*** gene telomeric to *ERBB2* gene are frequently co-amplified with *ERBB2* (Ooi et al. 2019). ***TOP2A*** gene, at 17q21.2, is amplified in 25-40% of *ERBB2* amplified breast cancers. *TOP2A* is a direct molecular target of anthracycline drug action and several studies have shown that *TOP2A* amplification is a marker of sensitivity for anthracyclines (Nielsen et al. 2008). Moreover, it has reported that loss of *TOP2A* is a significant prognostic factor for poor survival in breast cancer (Bartlett et al. 2010). ***BIRC5*** gene amplification, at 17q25.3, is suggested to predict distant recurrence in breast carcinoma (Davis et al. 2007).					
226	09628-L21977	***CPD***	17q11.2	CCAGTGACTACT-TACAAAACTGGA	9.0 **M**b
352	09963-L21558	***MED1***	17q12	TATCTCACACCA-AGGAGTGGGGGT	277.2 kb
148	00675-L18842	***ERBB2***, ex 3 (8)	NM_004448.4; 423-424	GGTGCAGGGCTA-CGTGCTCATCGC	4.1 kb
202	17591-L12908	***ERBB2***, ex 9 (14)	NM_004448.4; 1286-1287	GCAAGAAGATCT-TTGGGAGCCTGG	5.0 kb
316	00986-L28769	***ERBB2***, ex 15 (20a)	NM_004448.4; 2021-2022	CCATCTGGAAGT-TTCCAGATGAGG	9.2 kb
250	12048-L21551	***ERBB2***, ex 24 (29)	NM_004448.4; 3079-3080	TGTCGGCCAAGA-TTCCGGGAGTTG	562.9 kb
196	08611-L13204	***CDC6***	17q21.2	GAACCAACAAAT-GTCCAAACCGTA	113.4 kb
330	11999-L21541	***TOP2A***, ex 20	NM_001067.4; 2482-2483	AGTTTGGTACCA-GGCTACATGGTG	4.0 kb
365	12000-L28949	***TOP2A***, ex 14	NM_001067.4; 1764-1765	AAAGGCTTGCTG-ATTAATTTTATC	1.6 kb
172	11994-L12822	***TOP2A***, ex 11	NM_001067.4; 1374-1375	CAAGTCCAGTTA-AACAAGAAGTGT	5.6 **M**b
418	20778-L28948	***MAPT***	17q21.31	TAAAACCTTGAA-AAATAGGCCTTG	14.6 **M**b
208	22339-L21557	***PPM1D***	17q23.2	TGTGGTCATCAT-TCGGGGCATGAA	17.7 **M**b
323	03189-L02540	***BIRC5***, ex 1	NM_001168.3; 132-133	CTCTACATTCAA-GAACTGGCCCTT	0.4 kb

Length (nt)	SALSA MLPA probe	Gene/Exon[a]	Location / Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
436 «	03717-L28768	***BIRC5***, ex 2	NM_001168.3; 242-243	AGTGTTTCTTCT-GCTTCAAGGAGC	1.9 kb
392 ± «	03025-L28946	***BIRC5***, ex 3 (4)	NM_001168.3; 312-313	GCATTCGTCCGG-TTGCGCTTTCCT	-
CCNE1 gene, 19q12. *CCNE1* is often amplified in breast cancer cells and *CCNE1* overexpression has been associated with an increased risk of breast cancer relapse (Keyomarsi et al. 2002) and with resistance to trastuzumab (Scaltriti et al. 2011).					
190 «	09170-L09344	***CCNE1***, ex 6	NM_001238.4; 539-540	GGAAGTCTGGAA-AATCATGTTAAA	5.1 kb
166 «	02881-L02348	***CCNE1***, ex 11	NM_001238.4; 1187-1188	GATGGTTCCATT-TGCCATGGTTAT	-
AURKA gene, 20q13.2. 20q13 is frequently amplified in breast carcinoma samples and one of the putative target genes of the amplicon is the *AURKA* gene. High-level 20q13 amplifications, including *AURKA*, have been suggested to be an indicator of poor clinical outcome in breast cancer (Tanner et al. 1995).					
484	17590-L21028	***AURKA***, ex 7 (9)	NM_198437.3; 905-906	GCTCCATCTTCC-AGGTATGTAACT	9.8 kb
238	17365-L21549	***AURKA***, ex 5 (7)	NM_198437.3; 435-436	CTAGGAGGCAGT-GGGCTTTGGAAG	-

[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.

± SNPs rs187862045, rs767507216 and rs371975672 could influence probe signals at 177 nt, 268 nt and 392 nt, respectively. 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.

∫ Name of *C11ORF30* gene has been updated to *EMSY* according to the HUGO nomenclature throughout the document.

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.

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 tables above. Single probe aberration(s) must be confirmed by another method.

Table 3. Reference probes arranged according to chromosomal location.

Length (nt)	SALSA MLPA probe	Gene	Chromosomal band (hg18)	Partial sequence (24 nt adjacent to ligation site)	Location (hg18) in kb
373	05953-L30687	*SPAST*	2p22	GCAAGTTGTGCT-AGTTCTTTTTGG	02-032,222
143	14199-L15813	*EDAR*	2q13	GAGAGTTCTGTG-GGTGGAGAGAAG	02-108,894
346	03580-L02941	*SCN5A*	3p22	AAGATGATGAAA-ATGACAAAATAG	03-038,573
274	13796-L15290	*KCNAB1*	3q25	CTTTTCCAGAGA-GAGAAAGTGGAG	03-157,716
494	20779-L21727	*SNCA*	4q22	ACAGGAAGGAAT-TCTGGAAGATAT	04-090,869
184	10710-L11292	*PKHD1*	6p12	GGTTCCTGCTCT-TTCCAGTACCTC	06-052,016
127	15370-L19110	*POR*	7q11	GATGGGAAGTGA-GTGCCCACCCTG	07-075,448
409	01237-L27145	*UPF2*	10p14	TGCCATTCCTTT-GCATCTCAAAAG	10-012,019
220	13789-L31486	*LGI1*	10q23	TAGAGCTGAGTT-TCAATGACTATA	10-095,547
500	06676-L21510	*SMPD1*	11p15	CTGCTGAAGATA-GCACCACCTGCC	11-006,369
298	18378-L31470	*PKP2*	12p11	AGCCAGGCCAGA-TCATCTGGTCAG	12-032,840
385	09717-L28947	*NOS1*	12q24	GCTTGCAGATAT-GCATACAGCAGG	12-116,200
452	12459-L13460	*POMT2*	14q24	ATCACTGTGAAG-AACCTCCGGATG	14-076,832
310	09065-L09234	*CACNA1A*	19p13	CTCAGGCCTTCT-ACTGGACTGTAC	19-013,289

Complete probe sequences are available at www.mrcholland.com.

Related SALSA MLPA probemixes

- **P002 BRCA1:** contains probes for all exons of the *BRCA1* gene.
- **P045 BRCA2/CHEK2**: contains probes for all exons of the *BRCA2* gene and three probes for *CHEK2*, including one mutation-specific probe.
- **P087 BRCA1 Confirmation**: contains probes for all *BRCA1* exons.
- **P090 BRCA2**: identical to P045 BRCA2/CHEK2, but does not contain probes for *CHEK2*.
- **P175 Tumour Gain**: contains two probes for other exons of the *AURKA* gene.
- **P239 BRCA1 region**: contains *BRCA1* flanking probes on 17q21 (NBR1, NBR2 and BRCA1 pseudogene).
- **P315 EGFR**: contains 30 probes for the *EGFR* gene, including probes specific for the L858R and the T790M point mutations.
- **P370 BRAF-IDH1-IDH2**: contains six probes for the *FGFR1* gene.
- **P451 Chromosome 16**: contains 36 probes covering chromosome 16.
- **P483 HER gene family**: contains probes for the *EGFR*, *ERBB2*, *ERBB3* and *ERBB4* genes.

References

- Albertson D (2012). ESR1 amplification in breast cancer: controversy resolved? *J Pathol*. 227:1-3.
- Ali R et al. (2017). The paradoxical functions of EGFR during breast cancer progression. *Signal Transduction and Targeted Therapy*. 2:16042.
- Atanesyan L et al. (2017). Optimal fixation conditions and DNA extraction methods for MLPA analysis on FFPE tissue-derived DNA. *Am J Clin Pathol*. 147:60-8.
- Bartlett JM et al. (2010). Predictive markers of anthracycline benefit: a prospectively planned analysis of the UK National Epirubicin Adjuvant Trial (NEAT/BR9601). *Lancet Oncol.* 11:266-74.
- Chalmers IJ et al. (2001) Mapping the chromosome 16 cadherin gene cluster to a minimal deleted region in ductal breast cancer. *Cancer Genet Cytogenet.* 126:39-44.
- Chin K et al. (2006). Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. *Cancer Cell*. 10:529-41.
- Davis LM et al. (2007). Amplification patterns of three genomic regions predict distant recurrence in breast carcinoma. *J Mol Diagn.* 9:327-36.
- Deming SL et al. (2000). C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. *Br J Cancer*. 83:1688-95.
- Holland DG et al. (2011). ZNF703 is a common Luminal B breast cancer oncogene that differentially regulates luminal and basal progenitors in human mammary epithelium. *EMBO Mol Med*. 3:167–80.
- Holm K et al. (2012). Characterisation of amplification patterns and target genes at chromosome 11q13 in CCND1-amplified sporadic and familial breast tumours. *Breast Cancer Res Treat.* 133:583-94.
- Hömig-Hölzel C and Savola S. (2012). Multiplex ligation-dependent probe amplification (MLPA) in tumor diagnostics and prognostics. *Diagn Mol Pathol*. 21:189-206.
- Hu G et al. (2009). MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. *Cancer Cell.* 15:9-20.
- Keyomarsi K et al. (2002). Cyclin E and survival in patients with breast cancer. *N Engl J Med*. 347:1566-75.
- Kirkegaard T et al. (2008). Genetic alterations of CCND1 and EMSY in breast cancers. *Histopathology*. 52:698-705.
- Nielsen KV et al. (2008). The value of TOP2A gene copy number variation as a biomarker in breast cancer: Update of DBCG trial 89D. *Acta Oncol*. 47:725-34.
- Ooi A et al. (2012). Gene amplification of ESR1 in breast cancers-fact or fiction? A fluorescence in situ hybridization and multiplex ligation-dependent probe amplification study. *The Journal of Pathology*. 227:8-16.
- Ooi A et al. (2019). Amplicons in breast cancers analyzed by multiplex ligation-dependent probe amplification and fluorescence in situ hybridization. *Hum Pathol.* 85:33-43.
- Scaltriti M et al. (2011). Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. *Proc Natl Acad Sci U S A.* 108:3761-6.
- Park K et al. (2007). EGFR gene and protein expression in breast cancers. *Eur J Surg Oncol.* 33:956-60.
- 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.
- Singh JC et al. (2014). HER2-positive advanced breast cancer: optimizing patient outcomes and opportunities for drug development. *Br J Cancer.* 111:1888-98.
- Sircoulomb F et al. (2011). ZNF703 gene amplification at 8p12 specifies luminal B breast cancer. *EMBO Mol Med.* 3:153-66.
- Slamon DJ et al. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science.* 235:177-82.
- Tanioka M et al. (2014). Transcriptional CCND1 expression as a predictor of poor response to neoadjuvant chemotherapy with trastuzumab in HER2-positive/ER-positive breast cancer. *Breast Cancer Res Treat*. 147:513-25.
- Tanner MM et al. (1995). Amplification of chromosomal region 20q13 in invasive breast cancer: prognostic implications. *Clin Cancer Res.* 1:1455-61.
- Tokunaga E et al. (2014). Overexpression of metadherin/MTDH is associated with an aggressive phenotype and a poor prognosis in invasive breast cancer. *Breast cancer.* 21:341-9.
- Turner N et al. (2010). FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. *Cancer Res.* 70:2085-94.
- 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.
- Yang Z-Q et al. (2010). Transforming Properties of 8p11-12 Amplified Genes in Human Breast Cancer. *Cancer Research.* 70:8487-97.

Selected publications using SALSA MLPA Probemix P078 Breast tumour

- Ghaffari K et al. (2016). BIRC5 Genomic Copy Number Variation in Early-Onset Breast Cancer. Iran Biomed J. 20:241-5.
- Halilovic A et al. (2019). HER2, chromosome 17 polysomy and DNA ploidy status in breast cancer; a translational study. Sci Rep. 9:11679.
- Moelans CB et al. (2010). Molecular profiling of invasive breast cancer by multiplex ligation-dependent probe amplification-based copy number analysis of tumor suppressor and oncogenes. Modern Pathology. 23:1029-39.
- Moelans CB et al. (2011). Molecular differences between ductal carcinoma in situ and adjacent invasive breast carcinoma: a multiplex ligation-dependent probe amplification study. *Cell Oncol (Dordr).* 34:475-82.
- Moelans CB et al. (2014). Genomic evolution from primary breast carcinoma to distant metastasis: Few copy number changes of breast cancer related genes. *Cancer Lett.* 344:138-46.
- Moelans CB et al. (2018). Copy number changes at 8p11-12 predict adverse clinical outcome and chemo- and radiotherapy response in breast cancer. *Oncotarget.* 9:17078-92.
- Tabarestani S et al. (2014). Prognostic and predictive value of copy number alterations in invasive breast cancer as determined by multiplex ligation-dependent probe amplification. *Cell Oncol (Dordr).* 37:107-18.
- Verschuur-Maes AH et al. (2014). Analysis of gene copy number alterations by multiplex ligation-dependent probe amplification in columnar cell lesions of the breast. *Cell Oncol (Dordr).* 37:147-54.
  Vranic S et al. (2015). Immunohistochemical and molecular profiling of histologically defined apocrine carcinomas of the breast. *Hum Pathol.* 46:1350-59.

P078 product history	
Version	*Modification*
D2	Four reference probes were replaced and several probes have a small change in length, but no change in sequence detected.
D1	More than 40% of reference probes were replaced and/or added.
C2	Several probes have a small change in length, but no change in sequence detected. In addition, several reference probes were replaced and/or added.
C1	Two probes for *ZNF703* gene were included. In addition, several target, reference probes and 88 and 96 nt control fragments were replaced.
B1	Content completely revised. Target probes for *BIRC5* and *MTDH* added and several reference probes replaced.
A1	First release.

Implemented changes in the product description
Version D2-02 – 18 October 2022 (04P) - Product description restructured and adapted to a new template. - Various minor textual or layout changes. - Exon numbering of the *EMSY*, *ERBB2, EMSY, BIRC5* and *AURKA* genes has been changed in Table 2. - Transcript number of the *EMSY* and *BIRC5* genes has been changed in Table 2 according to the MANE Select transcripts. - Ligation sites of the probes targeting the *ESR1*, *FGFR1*, *MYC*, *CCND1* and *CDH1* genes updated according to new version of the NM_reference sequence. - List of related probemixes updated on page 10. - List of references has been updated. Version D2-01 – 11 April 2019 (01P) - Product description restructured and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - Various minor textual or layout changes. - Exon numbering of the *ESR1* gene has been changed in Table 2A. - Ligation sites of the probes targeting the *ZNF703, MYC, TOP2A, CCNE1* and *AURKA* genes updated according to new version of the NM_reference sequence. - Name of *C11ORF30* gene has been updated to *EMSY* according to the HUGO nomenclature throughout the document. - Warning added to Table 2a for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. - Warning removed for ERBB2 probe at 148 nt for SNP rs191376350, as the frequency of this SNP is very low (0.0024%). - For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36). - List of related probemixes updated on page 7.

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