SALSA MLPA probemix ME024-B2 9p21 CDKN2A/2B region

Lot B2-0615. As compared to the previous version B1 (lot B1-0411), one flanking probe is redesigned, two reference probes are replaced, and several probes have a small change in length but no change in the sequence detected.

For the detection of both copy number and methylation changes, the MS-MLPA protocol should be used.
In case only copy number changes are of interest, the standard MLPA protocol can be used.

Genomic losses of the 9p21.3 region, encompassing the CDKN2A/2B genes, are frequent events in many human cancers. This locus encodes three cyclin-dependent kinase inhibitors $p14^{ARF}$, $p16^{INK4A}$ and $p15^{INK4B}$ (see schematic presentation on page 2). Genomic deletion of one or both copies of these important cell cycle regulator genes is the main inactivation mechanism. CDKN2A deletion can extend to the MTAP gene, located 110 kb away. The MTAP gene encodes methylthioadenosine phosphorylase, an important enzyme for the salvage of both adenine and methionine. It is known that many tumour cells require addition of methionine to their growth medium, because their MTAP gene is co-deleted with CDKN2A. Cells lacking MTAP are expected to be sensitive to purine synthesis inhibitors and/or methionine starvation, and therefore homozygous co-deletion of the CDKN2A and MTAP genes might open possibilities for alternative treatment for cancer patients. Other genes that are frequently co-deleted with CDKN2A/2B are MIR31, CDKN2B-AS1 and PAX5. Loss of MIR31 has been shown to have pro-tumorigenic effects on e.g. breast and ovarian cancer (Creighton CJ et al. 2010, *Cancer Res*. 70:1906-15). CDKN2B-AS1 (non-protein coding CDKN2B antisense RNA 1) is suggested to act as an epigenetic silencer of the CDKN2B gene (Yu W et al. 2008*, Nature*. 451:202-6). The PAX5 gene, at 9p13, which is essential for normal B-cell lymphopoiesis, is frequently co-deleted with CDKN2A in B-ALL (Kim M et al. 2011, *Blood Cells Mol Dis*. 47:62-6).

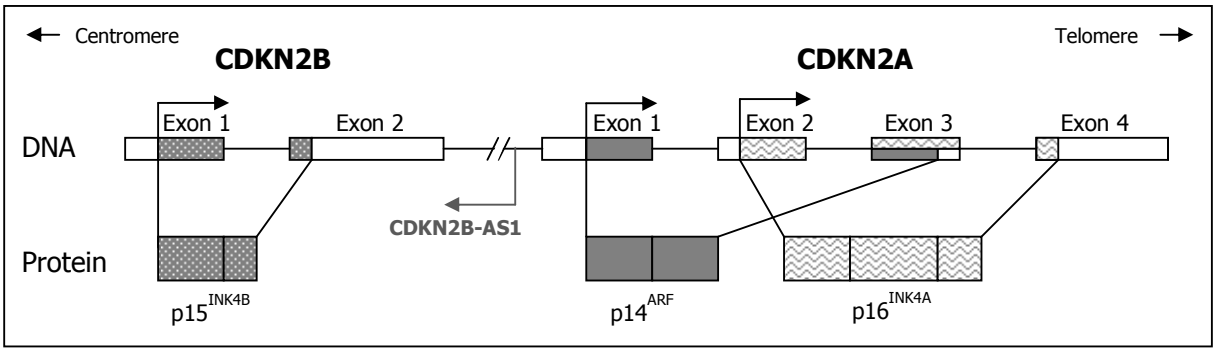
An alternative mechanism of inactivation of the CDKN2A/2B genes is the hypermethylation of the promoter regions leading to lack of expression of p14, p15 and p16 proteins, which further results in uncontrolled cell proliferation and tumour development and progression.

Alterations of the CDKN2A/2B genes are described not only in somatic tumour samples but also in the germline. Germline mutations in the CDKN2A gene have been linked to development of malignant cutaneous melanoma in some families with hereditary melanoma. Up to 40% of familial melanoma predisposition cases are associated with CDKN2A mutations (Hewitt C et al. 2002, *Hum Mol Genet*. 11:1273-9). Next to point mutations, various intragenic deletions within this gene have been identified in hereditary melanoma. The SALSA® MLPA® P419 Familial melanoma probemix contains more probes for CDKN2A/2B, and probes for CDK4. This P419 probemix can be used both on germline DNA and on tumour DNA.

This ME024-B2 MS-MLPA probemix contains 23 probes for the CDKN2A/2B gene region (including two probes for CDKN2B-AS1), ten of which are MS-MLPA probes which detect the methylation status of the promoter regions of these tumour suppressor genes. The MIR31, MTAP and PAX5 genes are covered by two probes each. Four more probes target the region between the MIR31 gene and the 9p telomere. Besides detecting aberrant methylation, all 33 target probes present will give information on copy number changes in the analysed sample. Additionally, 12 reference probes, not affected by HhaI digestion and located on stable genomic regions in most tumour types, and two digestion control probes that can be used to confirm complete digestion by the HhaI enzyme are included.

The MS-MLPA probes in this ME024-B2 probemix detect sequences in promoter regions of tumour suppressor genes that are unmethylated in most blood-derived DNA samples. Upon digestion, the peak signal obtained in unmethylated samples will be very small or absent. In contrast, when tested on *in vitro* methylated human DNA, these probes do generate a signal. We have no data showing that methylation detected by a particular probe indeed influences the corresponding mRNA levels.

Schematic presentation of CDKN2A and CDKN2B gene structure and proteins encoded by these genes



Note: An alternative exon 4 (330 nt before exon 4, present in NG_007485.1 sequence) was published in March 2011 and is present in NM_001195132.1 transcript variant 5. Clinical significance of this transcript variant is not yet known. This variant is also known as p16γ (Lin YC et al. 2007, *Oncogene.* 26:7017-27). Transcript variant 5 includes an additional exon that causes a frameshift in the 3' coding region when compared to variant 1 (encoding $p16^{INK4A}$). The resulting isoform p16γ has a distinct C-terminus, which is longer than $p16^{INK4A}$, and this isoform is a candidate for nonsense-mediated mRNA decay. However, it is not known if this endogenous protein p16γ is expressed *in vivo*.

This SALSA® MS-MLPA® probemix can be used to detect *aberrant methylation* of one or more sequences of promoter regions of the CDKN2A and CDKN2B genes. Methylation levels can be different for different tissues. If possible, use identically treated test and reference samples (same tissue type and extraction method). This SALSA® MS-MLPA® probemix can also be used to detect *copy number changes (deletions/duplications)* of one or more sequences in the above mentioned genes in a DNA sample. Heterozygous deletions of recognition sequences should give a 35-50% reduced relative peak height of the amplification product of that probe. Note that a mutation or polymorphism in the sequence detected by a probe can also cause a reduction in relative peak height, even when not located exactly on the ligation site! In addition, some probe signals are more sensitive to sample purity and small changes in experimental conditions. Therefore, deletions and duplications detected by MLPA should always be confirmed by other methods. Not all deletions and duplications detected by MLPA will be pathogenic; users should always verify the latest scientific literature when interpreting their findings. Finally, note that most defects in this gene are expected to be small (point) mutations which will not be detected by this SALSA® MS-MLPA® test. We have no information on what percentage of defects in these genes is caused by deletions/duplications of complete exons.

SALSA® MS-MLPA® probemixes and reagents are sold by MRC-Holland for research purposes and to demonstrate the possibilities of the MLPA technique. They are not CE/FDA certified for use in diagnostic procedures. Purchase of the SALSA® MS-MLPA® test probemixes and reagents includes a limited license to use these products for research purposes.

The use of this SALSA® MS-MLPA® probemix and reagents requires a thermocycler with heated lid and sequence type electrophoresis equipment. Different fluorescent PCR primers are available. The MLPA technique has been first described in Nucleic Acid Research 30, e57 (2002). The MS-MLPA method for the detection of both copy numbers and methylation changes was described in Nucleic Acid Research 33, e128 by Nygren et al. 2005.

More information

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Related SALSA® MLPA® probemixes

- P419 CDKN2A/2B-CDK4: Contains more probes for the CDKN2A/2B, CDK4 and MTAP genes, and a mutation-specific probe for MITF involved in Familial melanoma.
- P088 Oligodendroglioma 1p-19q: Contains more probes for the CDKN2A/2B genes, the regions 1p and 19q, and mutation-specific probes for IDH1/2.

References of SALSA® MS-MLPA® probemix ME024 9p21 CDKN2A/2B region

- Braun M et al. (2016) Biallelic loss of CDKN2A is associated with poor response to treatment in pediatric acute lymphoblastic leukemia. *Leuk Lymphoma.* 18:1-10.
- Navarro L et al. (2015) Alteration of major vault protein in human glioblastoma and its relation with EGFR and PTEN status. *Neuroscience*. 297:243-51.
- Nicolae-Cristea AR et al. (2015) Diagnostic and prognostic significance of CDKN2A/CDKN2B deletions in patients with transformed mycosis fungoides and primary cutaneous CD30-positive lymphoproliferative disease. *Br J Dermatol*. 172:784-8.
- de Ávila AL et al. (2014) Germline CDKN2A mutations in Brazilian patients of hereditary cutaneous melanoma. *Fam Cancer*. 13:645-9.
- Cabanillas R at al. (2013) Novel germline CDKN2A mutation associated with head and neck squamous cell carcinomas and melanomas. *Head Neck*. 35:80-4.
- Gardiner RB et al. (2012) Using MS-MLPA as an efficient screening tool for detecting 9p21 abnormalities in pediatric acute lymphoblastic leukemia. *Pediatr Blood Cancer*. 58:852-9.
- Meijer D et al. (2012) Genetic characterization of mesenchymal, clear cell, and dedifferentiated chondrosarcoma. *Genes Chromosomes Cancer*. 51:899-909.
- Conway C et al. (2010) Deletion of chromosome arm 9p in relation to BRAF/NRAS mutations and prognostic significance for primary melanoma. *Genes Chromosomes Cancer*. 49:425-38.
- Mohseny AB et al. (2010) Small deletions but not methylation underlie CDKN2A/p16 loss of expression in conventional osteosarcoma. *Genes Chromosomes Cancer.* 49:1095-103.
- Cesinaro AM et al. (2010) Alterations of 9p21 analysed by FISH and MLPA distinguish atypical spitzoid melanocytic tumours from conventional Spitz's nevi but do not predict their biological behaviour. *Histopathology.* 57:515-27.
- Binni F et al. (2010) Novel and recurrent p14 mutations in Italian familial melanoma. *Clin Genet*. 77:581-6.
- Ottaviano L et al. (2010) Molecular characterization of commonly used cell lines for bone tumor research: a trans-European EuroBoNet effort. *Genes Chromosomes Cancer.* 49:40-51.
- Barbaro M et al. (2009), Characterization of deletions at 9p affecting the candidate regions for sex reversal and deletion 9p syndrome by MLPA. *Eur J Hum Genet.* 17:1439-47.
- Freedberg DE et al. (2008) Frequent p16-independent inactivation of p14ARF in human melanoma. *J Natl Cancer Inst*. 100:784-95.
- Lesueur F et al. (2008) The contribution of large genomic deletions at the CDKN2A locus to the burden of familial melanoma. *Br J Cancer.* 99:364-70.
- Vignoli M et al. (2008) Genomic rearrangements of the CDKN2A locus are infrequent in Italian malignant melanoma families without evidence of CDKN2A/CDK4 point mutations. *Melanoma Res.* 18:431-7.
- Knappskog S et al. (2006) A novel type of deletion in the CDKN2A gene identified in a melanoma-prone family. *Genes Chromosomes Cancer*. 45:1155-63.
- Worsham MJ et al. (2006) Fine-mapping loss of gene architecture at the CDKN2B (p15INK4b), CDKN2A (p14ARF, p16INK4a), and MTAP genes in head and neck squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg*. 132:409-15.
- Mistry SH et al. (2005) Prevalence of 9p21 deletions in UK melanoma families. *Genes Chromosomes Cancer*. 44:292-300.
- Takata M et al. (2005) Genome profiling of melanocytic tumors using multiplex ligation-dependent probe amplification (MLPA): Its usefulness as an adjunctive diagnostic tool for melanocytic tumors. *J Dermatol Sci.* 40:51-7.
- Aveyard JS and Knowles MA. (2004) Measurement of relative copy number of CDKN2A/ARF and CDKN2B in bladder cancer by real-time quantitative PCR and multiplex ligation-dependent probe amplification. *J Mol Diagn*. 6:356-65.

Methylation-specific MLPA

Please note that each MS-MLPA reaction generates two samples that need analysis by capillary electrophoresis: one undigested sample for copy number detection and one digested sample for methylation detection.

A modification of the MLPA technique, MS-MLPA allows the detection of both copy number changes and unusual methylation levels of 10-50 different sequences in one simple reaction. MLPA probes for methylation quantification are similar to normal MLPA probes, except that the sequence detected by the MS-MLPA probe contains the sequence recognised by the methylation-sensitive restriction enzyme HhaI.
Similar to ordinary MLPA reactions, the MS-MLPA protocol starts with sample DNA denaturation and overnight hybridization. The reaction then is split into two tubes. One tube is processed as a standard MLPA reaction. This reaction provides information on copy number changes. The other tube of the MLPA hybridization reaction is incubated with the methylation-sensitive HhaI endonuclease while simultaneously, the hybridised probes are ligated. Hybrids of (unmethylated) probe oligonucleotides and unmethylated sample DNA are digested by the HhaI enzyme. Digested probes will not be exponentially amplified by PCR and hence will not generate a signal when analysed by capillary electrophoresis. In contrast, if the sample DNA is methylated, the hemi-methylated probe-sample DNA hybrids are prevented from being digested by HhaI and the ligated probes *will* generate a signal.

The MS-MLPA technique should always be internally validated before use in your laboratory. Results of MS-MLPA are highly dependent on the HhaI enzyme used. HhaI enzymes that are resistant to heat inactivation are NOT compatible with the MS-MLPA technique and will give aberrant results. These include, but may not be limited to, Thermo Fisher Scientific enzymes HhaI, ANZA 59 HhaI, and FastDigest HhaI. We recommend using Promega's HhaI enzyme (R6441) as this is the only restriction enzyme that has been validated for use with MS-MLPA by MRC-Holland.

More information about MS-MLPA can be found in the MS-MLPA protocol.

Please note that this product can <u>not</u> be used with an alternative protocol in which the genomic DNA is first digested with HhaI, followed by MLPA reactions on both digested and undigested genomic DNA.

Digestion control probes

The target sequences of the digestion control probes are unmethylated in most blood-derived DNA samples. The signals of the digestion control probes should be gone upon complete digestion by HhaI.

Data analysis

The ME024-B2 9p21 CDKN2A/2B region probemix contains 47 MLPA probes with amplification products between 122 nt and 499 nt. In addition, it contains 9 control fragments generating an amplification product smaller than 120 nt: four DNA Quantity fragments (Q-fragments) at 64-70-76-82 nt, three DNA Denaturation control fragments (D-fragments) at 88-92-96 nt, one X-fragment at 100 nt and one Y-fragment at 105 nt. More information on how to interpret observations on these control fragments can be found in the MLPA protocol.

The analysis of MS-MLPA probemixes consists of two parts: 1) determining copy numbers by comparing different undigested samples, and 2) determining methylation patterns by comparing each undigested sample to its digested counterpart (MS-MLPA probemixes only). The second part is unique for MS-MLPA probemixes and serves to semi-quantify the percentage of methylation within a given sample.

<u>1) Copy number analysis</u>

- Selection of reference probes
First select suitable reference probes for copy number detection. These are probes detecting relatively quiet regions in the particular type of tumour studied. The reference probes selected will therefore depend on the application. Probes that are suitable to use for reference in many types of tumour are indicated in Table 1.

- Intra-sample data normalisation
For analysis of MLPA results, not the absolute fluorescence values but "intra-normalised" data are used (relative peak heights). The data generated in the undigested sample should first be normalised intra-sample by dividing the signal of each probe by the signal of every reference probe in that sample, thus creating as many ratios per probe as there are reference probes. Subsequently, the median of all these produced ratios per probe should be taken; this is the probe's Normalisation Constant. This Normalisation Constant can then be used for sample to reference sample comparison.

- Inter-sample normalisation (comparison with reference samples)
The final probe ratio, or ploidy status, of each probe in each sample is calculated by dividing a) the Normalisation Constant of each probe obtained on the undigested test sample by b) the average Normalisation Constant of that probe obtained on the undigested reference samples.

2) Methylation analysis

- Selection of reference probes
Use the reference probes for methylation as marked in Table 1. All reference probes used for methylation analysis do not contain a HhaI site.

- Intra-sample data normalisation
For analysis of MLPA results, not the absolute fluorescence values but "intra-normalised" data are used (relative peak heights). The data generated in the digested sample should first be normalised intra-sample by dividing the signal of each probe by the signal of every reference probe in that sample, thus creating as many ratios per probe as there are reference probes. Subsequently, the median of all these produced ratios per probe should be taken; this is the probe's Normalisation Constant. This Normalisation Constant can then be used for sample to reference sample comparison.

- Methylation analysis (comparison with reference samples)
The methylation status of each MS-MLPA probe* in each sample is calculated by dividing a) the Normalisation Constant of each probe obtained on the digested test sample by b) the Normalisation Constant of each MS-MLPA probe obtained on the corresponding undigested sample. Multiplying this value by 100 gives an estimation of the percentage of methylation. Aberrant methylation can then be identified by comparing the methylation status of one or more MS-MLPA probes in the sample in question to that obtained on reference samples.

***Note:** An MS-MLPA probe targets a single specific HhaI site in a CpG island; if methylation is absent for a particular CpG-site, this does not necessarily mean that the whole CpG island is unmethylated!

Data normalisation should be performed within one experiment. Only samples purified by the same method should be compared. Confirmation of most exons deletions and amplifications can be done by e.g. Southern blotting, long range PCR, qPCR, FISH.

Warning: 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. Furthermore, there is always a possibility that one or more reference probes *do* show a copy number alteration in a sample. Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. When in doubt, users should always verify the latest updates of the database and scientific literature when interpreting their findings.

Note that Coffalyser, the MLPA analysis tool developed at MRC-Holland, can be downloaded free of charge from our website www.mlpa.com.

This probemix was developed at MRC-Holland.

Info/remarks/suggestions for improvement: info@mlpa.com.

Table 1. SALSA MLPA ME024-B2 9p21 CDKN2A/2B region probemix

Length (nt)	SALSA MLPA probe	HhaI site	% expected signal reduction	Chromosomal position			
				Reference	CDKN2A	CDKN2B	Other
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA						
88-92-96	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation						
100	X-fragment: Specific for the X chromosome						
105	Y-fragment: Specific for the Y chromosome						
122 ∫	Reference probe 02844-L02274			18q11			
130	**CDKN2B** probe 11867-L12664	+	100%			**Exon 1**	
136	**CDKN2A** probe 11868-L13885	+	100%		**upstream**		
143	Reference probe 14199-L15813			2q12			
151	**CDKN2A** probe 08658-L13470				**Intron 1**		
157	**CDKN2A** probe 16065-L18952	+	100%		**upstream**		
164 ¥	**MLLT3** probe 16058-L28698						9p21.3
170	**CDKN2A** probe 11869-L12666	+	100%		**Exon 2**		
178	**CDKN2B-AS1** probe 15671-L17637						9p21.3
184 *	Reference probe 18767-L24189			10q22			
190 ¥	**CDKN2B-AS1** probe 15672-L28771	+	100%				9p21.3
197	**CDKN2B** probe 16066-L00960					**Exon 1**	
209	**CDKN2B** probe 15673-L17639	+	100%			**Exon 1**	
220	**CDKN2B** probe 11871-L13741	+	100%			**Exon 1**	
229	**CDKN2B** probe 16059-L18233					**Exon 2**	
237	**CDKN2A** probe 01289-L00834	+	100%		**Exon 2**		
244	**CDKN2A** probe 16060-L19858				**Exon 2**		
250	Reference probe 07592-L19744			21q21			
259 ¥	**CDKN2A** probe 15674-L28708				**Exon 3**		
265 ¥	**CDKN2A** probe 15675-L19896				**Exon 4**		
273 ¥ +	**CDKN2A** probe 02238-L28709				**upstream**		
280 ¥	**CDKN2A** probe 16533-L28710				**Exon 4**		
287 ¥	**PAX5** probe 16061-L28711						9p13.2
293 ¥	**MTAP** probe 15677-L28712						9p21.3
301 ¥	Reference probe 07127-L28713			2p22			
312 ¥	**MTAP** probe 01293-L28714						9p21.3
319	**DOCK8** probe 01130-L00688						9p24.3
328 *	Reference probe 09065-L28777			19p13			
339 *	**GLDC** probe 20716-L26930						9p24.1
346	Reference probe 07402-L07049			12q13			
356	**CDKN2A** probe 01528-L06031				**Intron 1**		
363	**KLHL9** probe 16746-L19357						9p21.3
373	Reference probe 05288-L04644			14q22			
382	**MIR31** probe 16062-L18236						9p21.3
391	**CDKN2A** probe 08659-L11995				**Exon 2**		
400 #	**Digestion control** probe 10357-L10895	+	100%				22q12.3
409	Reference probe 08153-L08033			5q22			
416	**CDKN2B** probe 16064-L18238	+	95%			**Exon 1**	
426	**CDKN2A** probe 15680-L19745	+	100%		**Exon 1**		
436 # «	**Digestion control** probe 09167-L09460	+	100%				11q13.1
449 ¥	Reference probe 09107-L28897			4q25			
457 ¥	**CDKN2B** probe 20565-L28898					**downstream**	
465 ‡	**MIR31** probe 13665-L15119	+	0%				9p21.3
472	**CDKN2A** probe 16536-L19026				**Intron 1**		
480	**PAX5** probe 16063-L18237						9p13.2
490 ¥	Reference probe 12720-L28715			15q11			
499	Reference probe 06676-L19131			11p15			

* New in version B2 (lot B2-0615).
¥ Changed in version B2 (lot B2-0615). Small change in length, no change in the sequence detected.
Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
∫ Be cautious when interpreting results if the signal of this probe in the digested reaction is more than 10% lower than expected. A more than 20% lower signal in digested reactions of this probe indicates the use of an excess HhaI enzyme or the use of an enzyme preparation that is unsuitable for MS-MLPA such as the Thermo Fisher HhaI enzymes.
+ In several patients from the Netherlands and Belgium, a 6-bp deletion (GTACGC) in the target sequence of this CDKN2A probe (02238-L28709) has been reported. Please note that the pathological significance of this deletion is not clear.
‡ This MIR31 probe (13665-L15119) is not located in a CpG island. Nevertheless, it has an HhaI site but in our tests it never showed signal reduction upon HhaI digestion on DNA extracted from normal blood DNA; in tumour DNA we have no evidence if this HhaI site is methylated or not.
« This probe is located within, or close to, a very strong CpG island. A low signal of this probe can be due to incomplete sample DNA denaturation, e.g. due to the presence of salt in the sample DNA.

Notes

- **The CDKN2A/2B exon numbering has changed.** From description version 21 onwards, we have adopted the NCBI exon numbering that is present in the NG_007485.1 sequence for CDKN2A and in the NG_023297.1 sequence for CDKN2B. This exon numbering used here may differ from literature! The exon numbering used in previous versions of this product description can be found between parentheses in Table 2.
- The identity of the genes detected by the reference probes is available in Table 2. Please notify us of any mistakes: info@mlpa.com.

Table 2. ME024 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene / exon	Location / Ligation site	MV location (HG18)	HhaI site	Distance to next probe
Reference probes						
301	07127-L28713	SPAST	2p22.3	02-032.141981		76.8 **M**b
143	14199-L15813	EDAR	2q12.3	02-108.893937		-
449	09107-L28897	CFI	4q25	04-110.886835		-
409	08153-L08033	WDR36	5q22.1	05-110.455845		-
CDKN2A/CDKN2B at 9p21.3 and flanking probes						
319	01130-L00688	DOCK8, ex 23	NM_203447.3, 2924-2925	09-000.376335		6.2 **M**b
339	20716-L26930	GLDC, ex 20	NM_000170.2, 2644-2643 *reverse*	09-006.543336		13.8 **M**b
164	16058-L28698	MLLT3, ex 7	NM_004529.3, 1568-1569	09-020.353484		968.4 **M**b
363	16746-L19357	KLHL9, ex 1	NM_018847.2, 3553-3554	09-021.321836		180.2 kb
465 ‡	13665-L15119	MIR31 / MIR31HG	NR_029505.1, 45 nt after MIR31	09-021.502034	+	0.1 kb
382	16062-L18236	MIR31	NR_029505.1, 58-57 *reverse*	09-021.502096		342.7 kb
312	01293-L28714	MTAP, ex 6	NM_002451.3, 725-726	09-021.844754		4.5 kb
293	15677-L28712	MTAP, ex 7	NM_002451.3, 825-826	09-021.849296		108.2 kb
265	15675-L19896	**CDKN2A**, ex 4 (5)	NM_000077.4, 182 nt after ex 4	09-021.957523		0.7 kb
280	16533-L28710	**CDKN2A**, ex 4 (5)	NM_000077.4, 33 nt before ex 4	09-021.958235		3.0 kb
259	15674-L28708	**CDKN2A**, ex 3	NM_000077.4, 45 nt before ex 3	09-021.961213		3.5 kb
237	01289-L00834	**CDKN2A**, ex 2 (2a)	NM_000077.4, 434-433 *reverse* 127 nt after ATG	09-021.964676	+	0.2 kb
391	08659-L11995	**CDKN2A**, ex 2 (2a)	NM_000077.4, 205-206	09-021.964894		0.1 kb
244	16060-L19858	**CDKN2A**, ex 2 (2a)	NM_000077.4, 138-139	09-021.964957		0.2 kb
170	11869-L12666	**CDKN2A**, ex 2 (2a)	NM_000077.4, 102 nt before ex 2, 408 nt before ATG	09-021.965200	+	6.2 kb
151	08658-L13470	**CDKN2A**, intron 1	NM_000077.4, 6330 nt before ex 2 *reverse*	09-021.971433		6.3 kb
356	01528-L06031	**CDKN2A**, intron 1	NM_058195.3, 6387 nt after ex 1 *reverse*	09-021.977717		1.8 kb
472	16536-L19026	**CDKN2A**, intron 1	NM_058195.3, 4610 nt after ex 1	09-021.979488		4.9 kb
426	15680-L19745	**CDKN2A**, ex 1	NM_058195.3, 76-77, 84 nt before ATG	09-021.984375	+	0.4 kb
190	15672-L28771	CDKN2B-AS1, ex 1	NR_003529.3, 32-33; NM_058195.3, 491 nt before ATG	09-021.984790	+	0.2 kb

Length (nt)	SALSA MLPA probe	Gene / exon	Location / Ligation site	MV location (HG18)	HhaI site	Distance to next probe
			(CDKN2A)			
178	15671-L17637	CDKN2B-AS1, ex 1	NR_003529.3: 210-211	09-021.984968		0.3 kb
136 £	11868-L13885	**CDKN2A**, upstream	NM_058195.3, 762 before ex 1, 922 nt before ATG	09-021.985219	+	0.1 kb
157 £	16065-L18952	**CDKN2A**, upstream	NM_058195.3, 829 nt before ex 1, 989 nt before ATG	09-021.985282	+	0.2 kb
273 +£	02238-L28709	**CDKN2A**, upstream	NM_058195.3, 1022 nt before ex 1, 1182 nt before ATG	09-021.985479		5.0 kb
457	20565-L28898	**CDKN2B**, downstream	NM_078487.2, 2341 nt after ex 2 *reverse*	09-021.990530		5.3 kb
229	16059-L18233	**CDKN2B**, ex 2	NM_078487.2, 1030-1031	09-021.995813		3.0 kb
209	15673-L17639	**CDKN2B**, ex 1 (1b)	NM_078487.2, 459-460, 96 nt after ATG	09-021.998813	+	0.1 kb
130	11867-L12664	**CDKN2B**, ex 1 (1b)	NM_078487.2, 327-328, 33 nt before ATG	09-021.998949	+	0.1 kb
220	11871-L13741	**CDKN2B**, ex 1 (1b)	NM_078487.2, 260-261, 100 nt before ATG	09-021.999018	+	0.3 kb
197	16066-L00960	**CDKN2B**, ex 1 (1b)	NM_078487.2, 83 nt before ex 1	09-021.999356		0.1 kb
416	16064-L18238	**CDKN2B**, ex 1 (1b)	NM_078487.2, 155 nt before ex 1, 515 nt before ATG	09-021.999434	+	15.0 **M**b
287	16061-L28711	PAX5, ex 5	NM_016734.2, 967-968	09-036.992699		18.0 kb
480	16063-L18237	PAX5, ex 2	NM_016734.2, 589-590	09-037.010655		-
Reference probes and digestion controls						
184	18767-L24189	NODAL	10q22.1	10-071.865495		-
499	06676-L19131	SMPD1	11p15.4	11-006.369209		58.0 **M**b
436 #«	09167-L09460	MEN1	11q13.1 – Digestion control probe	11-064.334539	+	-
346	07402-L07049	COL2A1	12q13.11	12-046.660699		-
373	05288-L04644	ATL1	14q22.1	14-050.159633		-
490	12720-L28715	SNRPN-HB2-85	15q11.2	15-022.858476		-
122 ∫	02844-L02274	NPC1	18q11.2	18-019.394343		-
328	09065-L28777	CACNA1A	19p13.2	19-013.288974		-
250	07592-L19744	ADAMTS5	21q21.3	21-027.228886		-
400 #	10357-L10895	TIMP3	22q12.3 – Digestion control probe	22-031.527654	+	-

+ In several patients from the Netherlands and Belgium, a 6-bp deletion (GTACGC) in the target sequence of this CDKN2A probe (02238-L28709) has been reported. Please note that the pathological significance of this deletion is not clear.
∫ Be cautious when interpreting results if the signal of this probe in the digested reaction is more than 10% lower than expected. A more than 20% lower signal in digested reactions of this probe indicates the use of an excess HhaI enzyme or the use of an enzyme preparation that is unsuitable for MS-MLPA such as the Thermo Fisher HhaI enzymes.
‡ This MIR31 probe (13665-L15119) is not located in a CpG island. Nevertheless, it has an HhaI site but in our tests it never showed signal reduction upon HhaI digestion on DNA extracted from normal blood DNA; in tumour DNA we have no evidence if this HhaI site is methylated or not.
£ This probe is located on putative shared promoter region of CDKN2A and CDKN2B-AS1 genes.
Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
« This probe is located within, or close to, a very strong CpG island. A low signal of this probe can be due to incomplete sample DNA denaturation, e.g. due to the presence of salt in the sample DNA.

Notes

- **The CDKN2A/2B exon numbering has changed.** From description version 21 onwards, we have adopted the NCBI exon numbering that is present in the NG_007485.1 sequence for CDKN2A and in the NG_023297.1 sequence for CDKN2B. This exon numbering used here may differ from literature! The exon numbering used in previous versions of this product description can be found between parentheses in Table 2.
- **CDKN2A:** The NM_000077.4 sequence represents transcript variant 1, is a reference standard in the NCBI RefSeqGene project and encodes the $p16^{INK4A}$ protein.
  The NM_058195.3 sequence represents transcript variant 4, is a reference standard in the NCBI RefSeqGene project and encodes the $p14^{ARF}$ protein.

The NM_001195132.1 sequence represents transcript variant 5 that includes an additional exon and encodes the longer p16γ isoform with a distinct C-terminus.
Exon numbering for CDKN2A is assigned according to the NG_007485.1 reference standard sequence.

- **CDKN2B:** The NM_078487.2 sequence represents transcript variant 2, is a reference standard in the NCBI RefSeqGene project and encodes the $p15^{INK4B}$ protein.
  Exon numbering for CDKN2B is assigned according to the NG_023297.1 reference standard sequence.
- Exon numbering used here may differ from literature! Please notify us of any mistakes: info@mlpa.com.

Table 3. Sequences detected by the ME024 probes on 9p24.3-p13.2

Length (nt)	Gene	Complete sequences detected by the probes, with HhaI site in grey
319	DOCK8	CCGATCGCAACTGCAGCCGAATGTCTTACT-ATTGCTCTGGCAGTAGTGATGCTCCAAGTTCACCTGCAGC
339	GLDC	CCTGCACACCTGCACATACTCCCAGGCCTCACCTTGATA-TAAGCCCAGGAAATGGGCAAGATGGAACTGGAGCCCCA
164	MLLT3	GGAATCAGATGAAGTGGAGGATAACGACAAT-GACTCTGAAATGGAGAGGCCTGTAAATAGAGGAGGCAGCC
363	KLHL9	CCTGGTGCTGTTCCAGTCCATGTGCATCCTGA-GCTGTGTGATCTGCCTCGAGGCTATGATCTGAGCAAGCAGG
465	MIR31	CCACCTGCATGCCAGTCCTTCGTGTA-TTGCTGTGTATGTGCGCCCTTCCTTGGATGTGGAT
382	MIR31	GCTGCTGTCAGACAGGAAAGATGGCAAT-ATGTTGGCATAGCAGGTTCCCAGTTCAACAGCTATGCCAGCATCTTG
312	MTAP	ACATGACCACAGTTCCAGAGGTGGTTCTT-GCTAAGGAGGCTGGAATTTGTTACGCAAGTATCGCCATGGC
293	MTAP	CTCTAGGTTTCGGTGGACCGGGTCTTAA-AGACCCTGAAAGAAAACGCTAATAAAGCCAAAAGCTTACTGCTCACT
265	CDKN2A	GCCGTGTCTCAAGATCGATGAAATGCGGTT-AAAATGATGAATAGAAACTCTAGGGGGACCTCATATCGATAGACTC
280	CDKN2A	GGATGTGCCACACATCTTTGACCTCAGGT-TTCTAACGCCTGTTTTCTTTCTGCCCTCTGCAGACATCCC
259	CDKN2A	GGCTCTACACAAGCTTCCTTTCCGTCA-TGCCGGCCCCCACCCTGGCTCTGACCATTCTGTTCTCTCT
237	CDKN2A	CTGGATCGGCCTCCGACCGTAAC-TATTCGGTGCGTTGGGCAGCGCCCCCGCCTCCAGCAGC
391	CDKN2A	CTCTTCCGCCAGCACCGGAGGAA-GAAAGAGGAGGGGCTGGCTGGTCACCAGAGGGTG
243	CDKN2A	CACTCGCTCACGGCGTCCCCTTGCCTGGAAAGAT-ACCGCGGTCCCTCCAGAGGATTTGAGGGACAGGGTCGG
171	CDKN2A	TGAACGCACTCAAACACGCCTTTGCT-GGCAGGCGGGGGAGCGCGGCTGGGAGCAGGGAGGC
148	CDKN2A	GCTCCCAGGTACAGCTGTGTTAAGCCTTCA-TAGATGAGTTCTAGAATAGGATGTTGGGTGCAATAGATAATTA
356	CDKN2A	GACTCTCCTCCCTGGGATCCAGTAAACTG-ACTCTAAACTTAAAATCTTACCTAAAATCCTGGACCTCAATTCA
472	CDKN2A	AGCCAGATGGAGACCCAAGAGTGTTGAA-AGGCCACGACTTCCCTCAGTTTCTCCATCTGGGGGTGCAG
427	CDKN2A	GTGCGTGGGTCCCAGTCTGCAGTTA-AGGGGGCAGGAGTGGCGCTGCTCACCTCTGGTGCCAAAGG
190	CDKN2B-AS1	AGCTACATCCGTCACCTGACACGGCCCTACCA-GGAACAGCCGCGCTCCCGCGGATTCTGGTGCTGC
178	CDKN2B-AS1	GGTCATCTCATTGCTCTATCCGCCAATCAGGA-GGCTGAATGTCAGTTTTGAACTAAAAGCCGCTCCGCTCCTCTTC
136	CDKN2A	ATGGGCTAGACACAAAGGACTCGGTGCT-TGTCCCAGCCAGGCGCCCTCGGCGACGCGGGCAG
157	CDKN2A	GGGGAAGAGGAAAGAGGAAGAAGCGCTCAGAT-GCTCCGCGGCTGTCGTGAAGGTTAAAACCGAAAATAAA
273	CDKN2A	GGAGGGGACATGGAGGGGGAGACCGGAGAGA-GAACGTACGCCGAGGAGTCAGGCGGCGGGATCAA
457	CDKN2B	TGAGAGGGTGCCTCTGTGCCCTAGGAAAGGT-GATAGAGCTTAGAAACTCAGAACTCAGATGGAATGAGGAGCCA
229	CDKN2B	AGCCTGTAAGCCTGCAAGCCTGTCTGAGA-CTCACAGGAAGGAGGAGCCGACCGGGAATAACCTTCCATACAT
210	CDKN2B	GACTAGTGGAGAAGGTGCGACAGCTC-CTGGAAGCCGGCGCGGATCCCAACGGAGTCAACCGTTTCGG
130	CDKN2B	TCGTTAAGTTTACGGCCAACGGTGGAT-TATCCGGGCCGCTGCGCGTCTGGGGGCTGCGGAATGC
220	CDKN2B	GAAGCTGAGCCCAGGTCTCCTAGGAAGGA-GAGAGTGCGCCGGAGCAGCGTGGGAAAGAAGGGAA
199	CDKN2B	CGGGCCTGGCCTCCCGGCGAT-CACAGCGGACAGGGGGCGGAGCCTAAGGGGGTGGGGAGAC
417	CDKN2B	CTCCCTGGCCCAGTCTCTGGCGCA-TGCGTCCTAGCATCTTTGGGCAGGCTTCCCCGCC
287	PAX5	CGTGACGCAGGTGTCCTCGGTGAGCACGGAT-TCGGCCGGCTCGTCGTACTCCATCAGCGGCA
480	PAX5	GCCAGAGGATAGTGGAACTTGCTCATCAA-GGTGTCAGGCCCTGCGACATCTCCAGGCAGCTTCGGGTCAGCCATGGTT

The HhaI sites are marked with grey. Ligation sites are marked with -

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
Please notify us of any mistakes: info@mlpa.com.

SALSA MLPA probemix ME024-B2 9p21 CDKN2A/2B region sample pictures

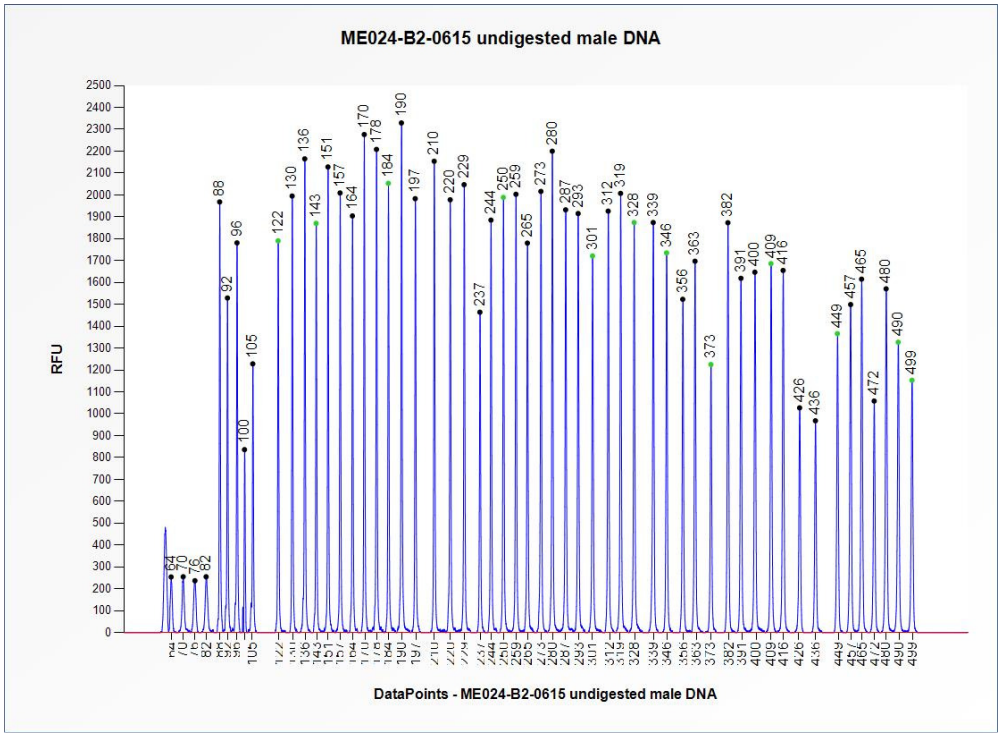

Figure 1. Capillary electrophoresis pattern of a sample of approximately 50 ng undigested human male control DNA analysed with SALSA MLPA probemix ME024-B2 9p21 CDKN2A/2B region (lot B2-0615) for the quantification of copy numbers.

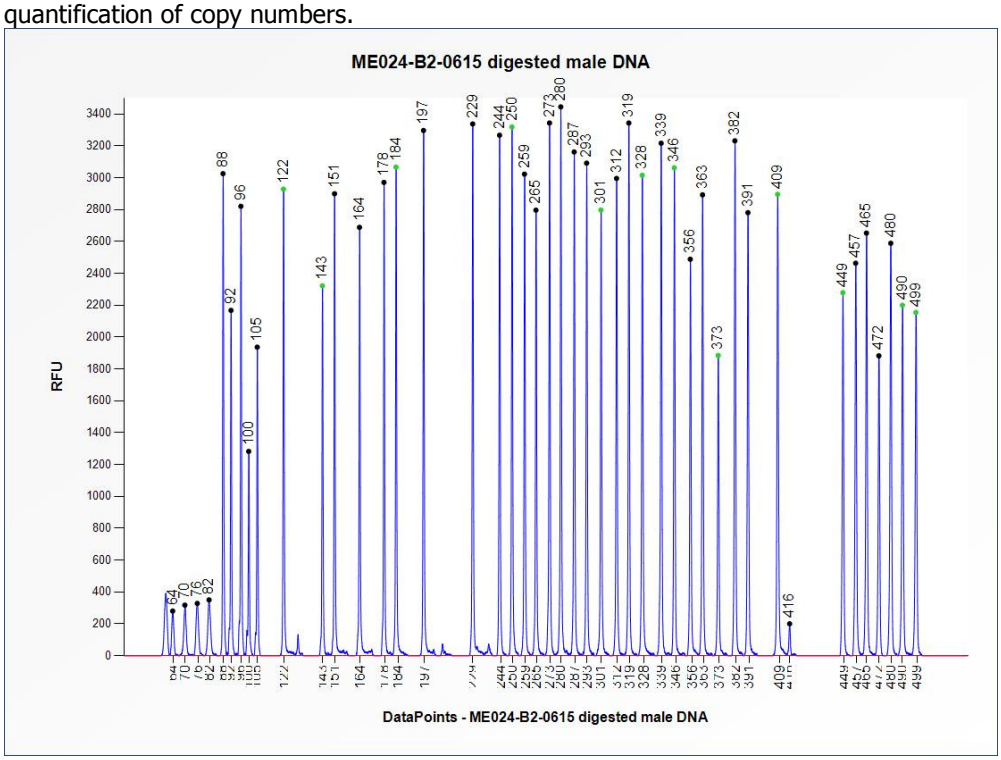

Figure 2. Capillary electrophoresis pattern of a sample of approximately 50 ng digested human male control DNA analysed with SALSA MLPA probemix ME024-B2 9p21 CDKN2A/2B region (lot B2-0615) to determine the methylation status.

Implemented Changes – the following has been altered compared to the previous product description version(s).

Version 23 – 26 January 2017 (16)
- Warning added in Table and Table 2 for the reference probe at 122 nt: decrease of >20% in the signal of this probes indicates use of excess Hha1 enzyme or use of an enzyme preparation unsuitable for MS-MLPA.
- Chromosomal positions in the header of Table 3 corrected.
- One new reference added on page 3.
- Various minor textual and layout changes.

Version 22 – 07 December 2016 (16)
- Warning regarding HhaI enzymes that are resistant to heat inactivation added under Methylation-specific MLPA section.

Version 21 – 29 July 2015 (14)
- Product description adapted to a new product version (version and lot number changed, changes in Table 1 and Table 2, new pictures included, various textual changes throughout the document).
- Warning added below Table 1 and 2 that the exon numbering used for CDKN2A and CDKN2B is updated according to the NG_007485.1 and NG_023297.1 sequences, respectively.
- New references added on page 3.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. For some probes, the length mentioned in Table 1 and Table 2 may differ slightly from previous product description versions, even when the probe has not undergone any physical changes. This is because Coffalyser.Net software is used for fragment analysis. If a probe HAS changed physically, this is mentioned explicitly.
- Ligation site of the probe targeting the MLLT3 gene is updated according to new version of the NM_ reference sequence.
- Schematic presentation on page 2 updated for the new CDKN2A and CDKN2B exon numbering, according to the NG_007485.1 and NG_023279.1 sequences, respectively.
- Note below schematic representation on page 2 updated according to the latest information on NM_001195132.1 transcript variant 5 coding for p16γ.

Version 20 (08)
- Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added.

Version 19 (08)
- One new reference added on page 2.
- Information added on page 6 for MIR31 probe (13665-L15119).
- Column with expected signal reduction % added to Table 1.
- Various minor textual and layout changes.

Version 18 (46)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Tables 1, 2 and 3, new pictures included).
- Information added on page 1 about CDKN2B-AS1, MIR31 and PAX5 genes.
- Reference articles have been added on page 2.
- Warning added on page 4 about the effects of low percentage of tumour cells for MLPA analysis.
- Picture on page 4 adapted for changes in exon numbering.
- Information added on page 4 about alternative exon 4.
- Various minor layout changes.

Version 17 (46)
- Product description adapted to a new lot (lot number added, small changes in Table 1 and 2, new picture included).
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.

Version 16 (06)
- Various minor textual changes on page 1, various minor layout changes, tables have been numbered.
- Data analysis section has been modified. For copy number analysis and methylation analysis different reference probes should be used (see Table 1).
- Website links to NCBI have been added under Table 3.
- A warning has been added about one of the CDKN2A probes (02238-L13277).