SALSA MLPA probemix P520-A2 MPN mix 2

Lot A2-0217. As compared to version A1 (lot A1-0115), several probes have been changed in length, but not in the sequence detected.

The **P520-A2 MPN mix 2** is an MLPA probemix that allows higher detection sensitivity for mutations. Only 1% allele burden is required for detection of the eight different mutations frequently found in MPN samples.

With each P520 probemix order, an artificial DNA sample (SD057) with an estimated 1% allele burden is supplied for data binning in the fragment analysis procedure, and as an artificial positive control for all the mutation-specific probes. This SD057 should be used in each P520 experiment for correct data binning and mutation calling.

Please note that this probemix should be used only as a qualitative tool (mutation present or absent) and NOT as a quantitative tool for measuring exact mutation burden. In addition, internal or external reference standards should be included both in the validation phase and in experiments.

This SALSA® MLPA® probemix is intended for research use only (RUO) purposes.

Note 1: The use of this product requires two modifications as compared to the General MLPA Protocol. First, the PCR reaction should be prolonged from 35 to 40 cycles. Second, we strongly recommend to use ≥100 ng of patient DNA per MLPA reaction (maximum 400 ng of DNA) for most optimal and robust mutation detection with the P520 probemix. Customers are strongly advised not to make any additional modifications to the MLPA protocol, as this could potentially lead to false positive or false negative results for the mutation calling.

Note 2: Probemix P420 MPN mix 1 can be used for patient samples with a >10% allele burden, which will generate a saturated mutation-specific signal when using P520.

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell malignancies, characterized by excessive production of blood cells. MPNs are subdivided in polycytemia vera (PV), essential thrombocytemia (ET), primary myelofibrosis (PMF) and less common conditions like chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), hypereosinophilic syndrome (HES) and mastocytosis.

Discovery of a frequent **JAK2** mutation (9p24.1), common to classic MPNs (PV, ET and PMF), has linked these diseases on a molecular level. The current WHO diagnostic criteria for classic MPNs include presence of JAK2, CALR or MPL mutations. The JAK2 V617F point mutation is detected in ~98% of PV patients, and in ~60% of patients with ET and PMF, whereas other JAK2 exon 12 mutations are commonly found in V617F negative PV patients. P520-A2 MPN mix 2 contains three mutation-specific JAK2 probes: one probe for **V617F** and two probes for the most common exon 12 mutations **N542_E543del** and **E543_D544del**.

Mutations in the **MPL** gene (1p34.2) are found in 4-11% of JAK2 V617F negative ET and PMF patients. P520-A2 MPN mix 2 contains two mutation-specific probes for MPL, **W515K** and **W515L**, that are diagnostically relevant in PV, ET and PMF according to the WHO classification.

The discovery of novel **CALR** gene (19p13.13) mutations in ET and PMF provides additional diagnostic tools for MPNs. Patients with ET and PMF but negative for JAK2 and MPL mutations, have been reported to harbour somatic insertions and deletions in exon 9 of the CALR gene. A 52-bp deletion (type 1) and a 5-bp insertion (type 2) are the most common mutations found in the CALR gene (53% and 32%, respectively). These mutations result in a frameshift to an alternative reading frame (Klampfl T et al. 2013, *N Engl J Med.* 369:2379-90; Nangalia J et al. 2013, *N Engl J Med.* 369:2391-405). CALR mutation-specific probes for the 52-bp deletion (**L367fs*46, type 1**) and 5-bp insertion (**K385fs*47, type 2**) are included in this probemix.

In addition, a probe specific for the **D816V** mutation in the **KIT** gene (4q12) is present. This is the most common KIT mutation and is present in >90% of patients with systemic mastocytosis (SM). Consequently, the presence of this mutation is considered a diagnostic criterion of SM according to the WHO classification.

Finally, 17 reference probes are included in the P520-A2 probemix, detecting different autosomal chromosomal locations which are relatively stable in MPNs. Please note that the probe signals of the reference probes in this P520 probemix show much greater variation as compared to standard MLPA products. This is due to the nature of the high sensitivity of this assay.

This SALSA® MLPA® probemix is designed to detect the presence of the aforementioned mutations in a DNA sample. 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 can be more sensitive to sample purity and small changes in experimental conditions.

This MLPA probemix cannot detect any mutations that are outside the target sequences of the MLPA probes. Neither can it be used for copy number detection of the aforementioned target genes. Even when MLPA analysis did not detect any aberrations, the possibility remains that changes in the corresponding gene(s) do exist but remain undetected. This probemix covers only the most frequent mutations of clinical and diagnostic relevance in MPNs. Rare or infrequent mutations are not covered. **If mutation calling with MLPA is negative for a patient sample, we recommend to perform sequencing of the JAK2, MPL and CALR genes to detect the rare or unknown mutations possibly present in a patient sample**.

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.

The use of this SALSA® 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 Acids Research 30, e57 (2002).

Related SALSA® MLPA® probemixes

- P420 MPN mix 1: This probemix is a conventional MLPA probemix allowing detection of the same mutations as included in P520 on samples with >10-20% allele burden (up to 100% allele burden).

More information

Website : www.mlpa.com
E-mail : info@mlpa.com (information & technical questions); order@mlpa.com (for orders)
Mail : MRC-Holland bv; Willem Schoutenstraat 1, 1057 DL Amsterdam, the Netherlands

Modifications to the One-Tube MLPA General Protocol

Please note that this P520 probemix should be used together with a modified MLPA general protocol with 40 PCR cycles. In addition, we recommend to use 100 ng of DNA as a minimum amount of sample DNA per MLPA reaction. Customers are strongly advised not to make any additional modifications to the MLPA protocol, as this could potentially lead to false positive or false negative results for the mutation calling. The standard One-Tube MLPA protocol should be followed, but using the following thermocycler program for the MLPA reaction:

a)	DNA denaturation		
1.	98°C	*5 minutes*	
2.	25°C	*pause*	
b)	Hybridisation reaction		
3.	95°C	*1 minute*	
4.	60°C	*pause*	
c)	Ligation reaction		
5.	54°C	*Pause*	
6.	54°C	*15 minutes*	
7.	98°C	*5 minutes*	
8.	20°C	*pause*	
d)	PCR reaction		
9.	**40 cycles**:	• 95°C	*30 seconds*
		• 60°C	*30 seconds*
		• 72°C	*60 seconds*
10.	72°C	*20 minutes*	
11.	15°C	*pause*	

Probemix content

The P520-A2 MPN mix 2 contains 25 MLPA probes with amplification products between 115 and 338 nt. In mutation-negative samples, only the 17 reference probes will generate a signal. The remaining eight probes will only generate a signal when the corresponding mutation is present in the patient sample. Please note however that mutation-specific probes can generate a low background signal in normal samples. Furthermore, literature suggests that some individuals in the general population can have mutation-positive clones at low levels (See e.g. Sidon P et al. 2006, *Leukemia*, 20:1622; Nielsen C et al. 2013, *Br J Haematol*. 160:70-9).

This probemix is modified to allow higher detection sensitivity for mutations. Only >1% allele burden is needed for reliable detection of the following mutations:

JAK2 p.V617F = c.1849G>T
JAK2 p.E543_D544del = c.1627_1632delGAAGAT
JAK2 p.N542_E543del = c.1624_1629delAATGAA
MPL p.W515L = c.1544G>T
MPL p.W515K = c.1543_1544TG>AA
KIT p.D816V = c.2447A>T
CALR p.L367fs*46 = c.1092_1143del52
CALR p.K385fs*47 = c.1154_1155insTTGTC

In addition to these 25 probes, this probemix contains nine 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.

In contrast to conventional MLPA probemixes, the Q-fragments in P520 will provide a warning in Coffalyser.Net already when less than 100 ng of sample DNA is used per reaction. See Figure 1 below for the effect of DNA quantity on the Q-fragments. The more sample DNA is used per reaction, the lower the Q-

fragments. Q-fragments are high when DNA amount is too low or ligation has failed. When all four Q-fragment have signals >33% of the 92 nt control fragment this indicates that DNA quantity was insufficient for the MLPA reaction (<100 ng) and Coffalyser.Net software will indicate a warning for that particular sample. Additional information on how to interpret observations on the D/X/Y-control fragments can be found in the MLPA protocol.

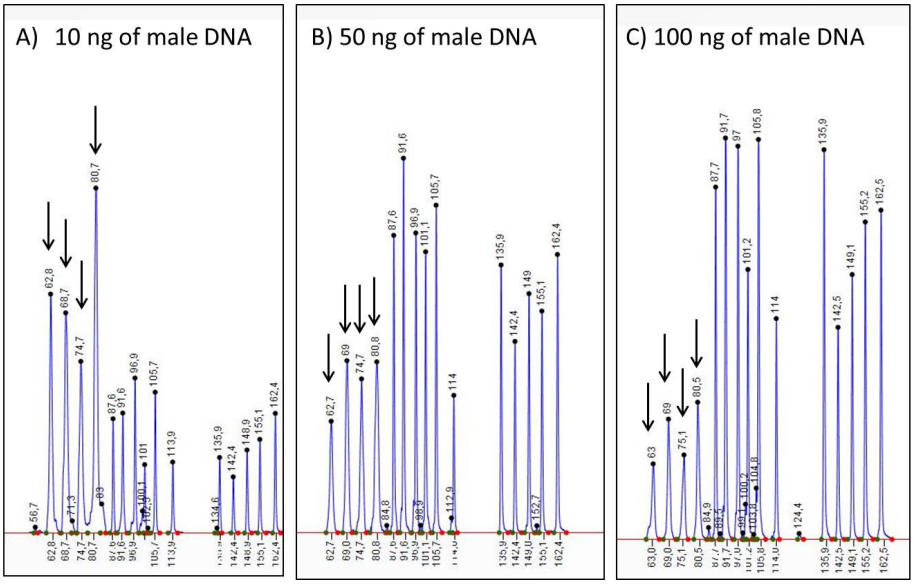


Figure 1. Q-fragments in the P520-A2 probemix. P520-A2 probemix analysed with A) 10 ng of male DNA; median of Q-fragments is higher than the 92 nt control fragment and in Coffalyser.Net analysis a red warning is indicated; B) 50 ng of male DNA; median of Q-fragments is higher than 33% of the 92 nt control fragment and in Coffalyser.Net an orange warning is indicated; C) 100 ng of male DNA, Q-fragments are <33% of the 92 nt probe and no warning is indicated in Coffalyser.Net). The Q-fragments are indicated with arrows. Note that the MLPA peak pattern of the P520-A2 probemix is shown here only partially, until 162 nt.

SD057 Sample DNA

The SD057 Sample DNA, provided with each P520-A2 probemix vial, is an aid for binning of the eight mutation-specific probes: CALR L367fs*46 (S0999-L26702; 124 nt), CALR K385fs*47 (S1001-L26517; 130 nt), JAK2 N542_E543del (16924-L21237; 167 nt), JAK2 E543_D544del (16924-L21238; 172 nt), MPL W515K (S1048-SP0405-L29870; 179 nt), MPL W515L (S1048-SP0405-L29871; 185 nt), KIT D816V (17722-SP0542-L23707; 200 nt) and JAK2 V617F (13190-L21572; 240 nt).

NOTE that this SD057 sample DNA contains an estimated 1% burden of the above mentioned mutation-specific sequences. Inclusion of one reaction with SD057 DNA in MLPA experiments is recommended as it can be used to aid in data binning of the peak pattern using Coffalyser.NET software and as an artificial positive control for the specific point mutations. SD057 DNA consists of female DNA mixed with a plasmid DNA that contains the target sequences detected by the above mentioned probes. MLPA reactions for binning purposes should be performed with 5 µl of SD057 (20 ng/µl) with total of 100 ng of DNA. **This product is for research use only (RUO).**

Data analysis

Data generated by this probemix should be normalised with a more robust method, as the target sites of the reference probes may be gained or lost in MPN patient samples. (1) Intra-sample normalisation should be performed by dividing the signal of each target-specific probe by the signal of every single reference probe in that sample, thus creating as many ratios per target-specific 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. (2) Secondly, inter-sample comparison should be performed by dividing the Normalisation Constant of each probe in a given sample by the average Normalisation Constant of that probe in all the reference samples.

Data normalisation should be performed within one experiment. Always use sample and reference DNA extracted with the same method and derived from the same source of tissue. Confirmation of the presence of a specific point mutation can be done by e.g. Sanger sequencing, next generation sequencing, qPCR, or digital droplet PCR. In particular, it is recommended to confirm positive mutation calls with low mutation burden with the above mentioned methods.

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

In addition to Coffalyser analysis, visual examination of the P520 MLPA peak patterns is necessary for mutation calling (see Figure 2 for negative mutation calling and Figure 4 for saturated mutation-specific MLPA peaks).

Interpretation of the results

-Mutation calls
Comparison of the relative peak height of a mutation-specific probe (vs. reference probes) on a positive sample with the relative peak height obtained with that probe on the SD057 sample DNA provides an indication of the presence for the corresponding mutation. SD057 contains an estimated 1% allele burden.

In case the signal of the mutation-specific probe in a sample of interest is higher than the signal of the corresponding probe in the SD057 reaction, this is considered to be a true positive call for the respective mutation. When the mutation-specific signal is lower than the corresponding mutation-specific probe signal in the SD057 reaction, this is regarded as a negative call (see example in Figure 2).

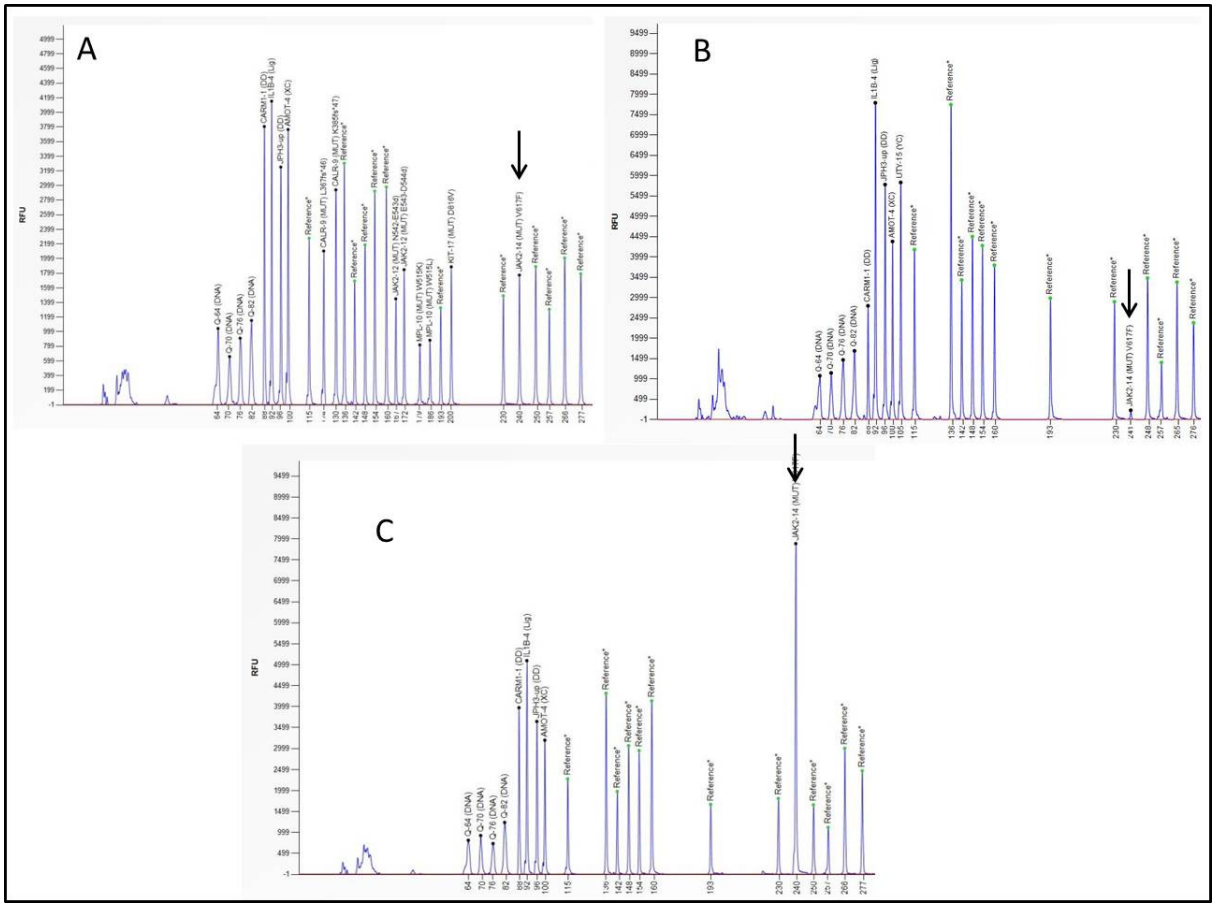


Figure 2. Mutation calling with the P520-A2 probemix: examples of negative and positive mutation calls for the JAK2 V617F point mutation. A) P520-A2 probemix analysed with SD057 sample DNA that contains an estimated 1% burden of the JAK2 V617F mutation sequence. The peak of the JAK2 V617F specific probe is indicated with an arrow. B) An example of a normal sample from a healthy individual that has a very small background peak at the length of the JAK2 V617F specific probe (at 240 nt, indicated with an arrow). As the height of this probe is lower than the height of the JAK2 V617F specific probe in SD057, this is considered a negative call for the JAK2 V617F point mutation. C) An example of a patient sample with 5% allelic burden for the JAK2 V617F mutation (peak at 240 nt, indicated with an arrow). As the height of this signal is higher in comparison with the JAK2 V617F signal in the SD057 sample DNA (A) this results can be considered as a positive call for the JAK2 V617F mutation. Please note that the MLPA peak pattern for the P520-A2 probemix is shown here only partially, until 276 nt.

We highly recommend the use of **negative control samples** from healthy individuals in the experiments to evaluate the background signal for the mutation-specific probes in the MLPA peak pattern for your specific types of samples and DNA extraction method used.

We highly recommend to also include **mutation positive samples (cell line or patient derived) extracted with the same DNA extraction method as the patient samples** in MLPA experiments to optimize mutation calling for your patient samples.

Sources for commercial **positive reference samples, tested by MRC-Holland**:

- Reference standard sample for the JAK2 p.V617F point mutation (Catalog IDs: HD649 for mutant and HD652 for WT) available at www.horizondiscovery.com (Horizon Discovery).
- JAK2 p.V617F WHO reference panel (code 16/120) (nominal values 100% [100%], 90% [89.5%], 30% [29.6%], 10% [10.8%], 1% [1.0%], 0.1% [0.03%] and 0% [0%] VAF – in square brackets is the proposed consensus value by WHO committee) - www.nibsc.org (The National Institute for Biological Standards and Control - NIBSC).
- Quan-Plex™ NGS Reference Standard Genomic DNA (Catalog ID: ARF-1001G-1 for mutant) including KIT p.D816V and Onco-Ref™ Genomic DNA Reference Standard HCT116 WT (Catalog ID: ASO-6052-1)

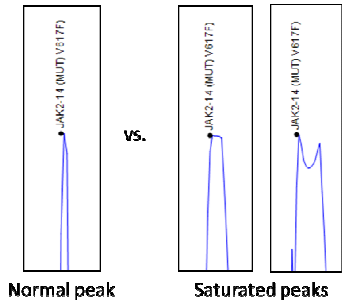
The following **positive reference standards** are also available, but have not been tested at MRC-Holland:

- Seraseq Myeloid Mutation DNA Mix (code 0710-0408) with CALR p.L367fs*46 (5% VAF), JAK2 p.V617F (5% VAF), MPL p.W515L (5% VAF) and JAK2 pN542_E543del (5% VAF) www.seracare.com (Sera Care).

-Saturated MLPA peak(s) with >5% allele burden samples

When a patient sample has >5% allele burden, the corresponding MLPA probe peak may become saturated in the MLPA peak pattern (see Figure 3 below for further information).

Figure 3. Normal vs. saturated MLPA peaks. Saturated peaks can be detected in the MLPA peak pattern by zooming in: a blunt end of an MLPA peak or alternatively a double peak at the very tip of the MLPA peak is then detected.



High allele burden (»5%) commonly results in an extra "shadow peak" ~15 nt before the real mutation-specific peak. This "shadow peak" has the exact same nucleotide sequence as the real MLPA peak, however, due to very high concentration of target sequence, is forming additional secondary structures that result in a faster running speed in the capillary electrophoresis and the formation of the "shadow peak" (see Figure 4 for further information).

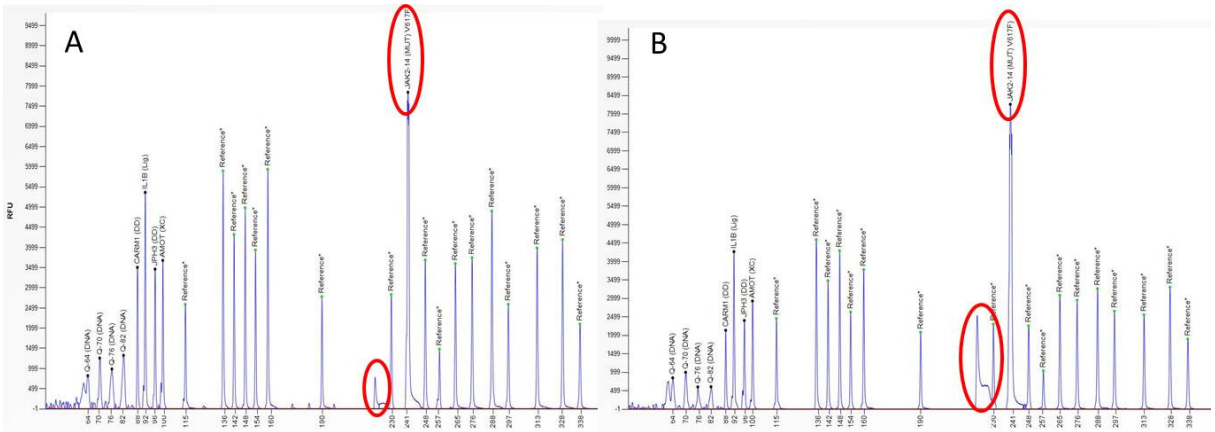


Figure 4. Saturated mutation-specific peaks in the MLPA peak pattern. A) JAK2 V617F positive patient sample with 10% allele burden was analysed with the P520-A2 probemix and run at an ABI sequencer. The JAK2 V617F specific peak at 240 nt is saturated and there is a "shadow peak" visible at 222 nt. B) The same JAK2 V617F positive cell line sample with 50% allele burden and run at the ABI sequencer showing an increased shadow peak at 222 nt.

When a "shadow peak" (the unspecific peak ~15 nt from the saturated mutation-specific peak) is detected in the MLPA peak pattern, a rerun on the capillary electrophoresis device using a dilution (e.g. 20x) of the PCR product with pure water can be used to lower this "shadow peak" (see Figure 4B). Please note that with this dilution the other probe signals in the MLPA peak pattern are diminished as well.

- Confirmation of >10-20% mutation burden calls with P420 MPN mix 1

We recommended to use P420 MPN mix 1 for patient samples when there is a saturated mutation-specific signal in P520 in case of >10 % allele burden present in the patient sample and in order to confirm the result. The P420 assay is a conventional MLPA assay allowing detection of mutations on samples with >10-20% allele burden (up to 100% allele burden). For samples with estimated 1-10% allele burden we recommend to use P520 as qualitative tool for mutation detection.

Warning: MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Mutations may not be detected if the percentage of tumour cells is low or if the allelic burden of the mutation is <1%. Furthermore, although reference probes are located in 'silent' regions that are not frequently altered in copy number in MPN patient samples, there is always a possibility that one or more reference probes *do* show a copy number alteration in a sample. When in doubt, users should always verify the latest updates of the databases and scientific literature when interpreting their findings.

This probemix was developed by M. Steenkamer, A. Stuitje and S. Savola at MRC-Holland. In case the results obtained with this probemix lead to a scientific publication, it would be very much appreciated if the probemix designers could be included as co-authors.

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

Table 1. SALSA MLPA P520-A2 MPN mix 2

Length (nt)	SALSA MLPA probe	Chromosomal position		
		Reference	Target	Mutation details
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			
115	Reference probe S0973-L26704	4p13		
124 §	**CALR probe** S0999-L26702		19p13.13	**p.L367fs*46**=c.1092_1143del52
130 §	**CALR probe** S1001-L26517		19p13.13	**p.K385fs*47**=c.1154_1155insTTGTC
136	Reference probe 16316-L25926	3q21		
142	Reference probe 07387-L26769	12q13		
148	Reference probe 10663-L11245	6p12		
154	Reference probe 13781-L15275	11p14		
160	Reference probe 17621-L21665	10q22		
167 § £	**JAK2 probe** 16924-L21237		9p24.1	**p.N542_E543del**=c.1624_1629delAATGAA
172 § £	**JAK2 probe** 16924-L21238		9p24.1	**p.E543_D544del**=c.1627_1632delGAAGAT
179 § Ж ¥	**MPL probe** S1048-SP0405-L29870		1p34.2	**p.W515K**=c.1543_1544TG>AA
185 § Ж ¥	**MPL probe** S1048-SP0405-L29871		1p34.2	**p.W515L**=c.1544G>T
193 ¥	Reference probe 11556-L26606	5q31		
200 § Ж	**KIT probe** 17722-SP0542-L23707		4q12	**p.D816V**=c.2447A>T
230	Reference probe 17130-L26574	11p11		
240 § Σ ¥	**JAK2 probe** 13190-L21572		9p24.1	**p.V617F**=c.1849G>T
250 ¥	Reference probe 05386-L29196	12p11		
257 ¥	Reference probe 13572-L29195	1q23		
265	Reference probe 12434-L26073	14q24		
277	Reference probe 16270-L26771	20q11		
287	Reference probe 05713-L20268	2p11		
297	Reference probe 04570-L20036	16q13		
313	Reference probe 04833-L20693	5p13		
328	Reference probe 13397-L26608	6q12		
338	Reference probe 12785-L15496	2q12		

¥ Changed in version A2 (from lot A2-0217 onwards). Small change in length, no change in sequence detected.
§ Mutation-specific probe. This probe will generate a signal when the mutation is present.
Ж This probe consists of three parts and has two ligation sites.
£ When probe signals at 167 nt and 172 nt both are present in the MLPA peak pattern, it is indicative for the JAK2 E543_D544del mutation. When only the probe signal at 167 nt is present, it is indicative for the JAK2 N542_E543del mutation.
Σ This probe can have a very low unspecific background signal detected also in healthy control samples. This background signal has never exceeded the signal detected in JAK2 V617F samples with an 1% allele burden in the internal quality tests at MRC-Holland. See Figure 2 for further information on this.

Note: Please notify us of any mistakes. The identity of the genes detected by the reference probes is available on request: info@mlpa.com.

Table 2. P520 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene / Exon / Mutation details	Ligation site	Partial sequence (24 nt adjacent to ligation site)	MV location (HG18)
MPL gene, at 1p34.2, indicated ligation sites are in NM_005373.2. Mutations in the thrombopoietin receptor (MPL) gene, most commonly W515L and W515K mutations in exon 10, are detected in patients with ET and PMF, which can support diagnosis when JAK2 is not mutated. W515L and W515K are reported to be the predominant MPL mutations in MPNs (Ma W et al. 2011, *Diagn Mol Pathol*, 20:34-9).					
185 § Ж	S1048-SP0405-L29871	**MPL**, exon 10 **p.W515L=c.1544G>T**	1589-1590 and 25nt after ex 10	GCTGCTGATGT**T**-46nt spanning oligo-TGGCGGTGGACC	01-043.587.563
179 § Ж	S1048-SP0405-L29870	**MPL**, exon 10 **p.W515K=c.1543_1544TG>AA**	1589-1590 and 25nt after ex 10	GCTGCTGAGG**AA**-46nt spanning oligo-TGGCGGTGGACC	01-043.587.563
KIT gene, at 4q12, indicated ligation sites are in NM_000222.2. Somatic activating KIT D816V mutation is present in more than 97% of systemic mastocytosis patient samples (Erben P et al. 2014, *Ann Hematol*, 93:81-8). This D816V mutation results in ligand-independent activation of KIT tyrosine kinase and detection of this point mutation aids in prediction of response to tyrosine kinase inhibitor (TKI) therapy (imatinib).					
200 § Ж	17722-SP0542-L23707	**KIT**, exon 17 **p.D816V=c.2447A>T**	2496-2495 and 2535-2534 *reverse*	CATTCTTGATG**A**-38nt spanning oligo-CCATGAGTAAGG	04-055.293.998
JAK2 gene, at 9q24.1, indicated ligation sites are in NM_004972.3. Discovery of a JAK2 mutation common to MF, PV and ET has linked these diseases on a molecular level and diagnostic criteria for MPN include detection of a clonal marker e.g. JAK2 V617F mutation in exon 14 or exon 12 mutations. JAK2 is mutated in 97% of all patients with PV, 55% of patients with ET and 65% of patients with PMF (Tefferi A, 2010, *Leukemia*, 24:1128–38). The most common JAK2 mutations in exon 12 are the following 6-bp deletions: N542_E543del and E543_D544del.					
167 § £	16924-L21237	**JAK2,** exon 12, **p.N542_E543del =c.1624_1629delAATGAA**	2126-2127	AAAATCAGAGAT-TTGATATTTGTA	09-005.059.976
172 § £	16924-L21238	**JAK2,** exon 12, **p.E543_D544del =c.1627_1632delGAAGAT**	2126-2127	AAAATCAGAAAT-TTGATATTTGTA	09-005.059.976
240 § ∑	13190-L21572	**JAK2,** exon 14 **p.V617F=c.1849G>T**	2343-2342 *reverse*	GTCTCCACAGA**A**-ACATACTCCATA	09-005.063.725
CALR gene, at 19p13.13, indicated ligation sites are in NM_004343.3. Majority of patients with ET or PM that are negative for JAK2 and MPL carry a somatic mutation in CALR gene. A 52-bp deletion (type 1=L367fs*46) and 5-bp insertion (type 2=K385fs*47) are the most common mutations found in CALR (53% and 32%, respectively), resulting in a frameshift to an alternative reading frame (Klampfl T et al. 2013, *N Engl J Med,* 369:2379-90; Nangalia J et al. *N Engl J Med,* 369:2391-405).					
130 §	S1001-L26517	**CALR**, exon 9**, p.K385fs*47 =c.1154_1155insTTGTC**	1234-1235	CAGAGGACAA**TT**-**GTC**GGAGGATGA	19-012.915.595
124 §	S0999-L26702	**CALR**, exon 9**, p.L367fs*46 =c.1092_1143del52**	1224-1223 *reverse*	CTTGTCCTCTGC-TCCTCGTCCTGT	19-012.915.528

§ Mutation-specific probe. This probe will generate a signal when the mutation is present.
Ж This probe consists of three parts and has two ligation sites.
£ When probe signals at 167 nt and 172 nt both are present in the MLPA peak pattern, it is indicative for the JAK2 E543_D544del mutation. When only the probe signal at 167 nt is present, it is indicative for the JAK2 N542_E543del mutation.
∑ This probe can have a very low unspecific background signal detected also in healthy control samples. This background signal has never exceeded the signal detected in JAK2 V617F samples with 1% allele burden in the internal quality tests at MRC-Holland. See Figure 2 for further information on this.

Note: Exon numbering may differ from literature! Complete probe sequences are available on request: info@mlpa.com. Please notify us of any mistakes: info@mlpa.com.

SALSA MLPA probemix P520-A2 MPN mix 2 sample pictures

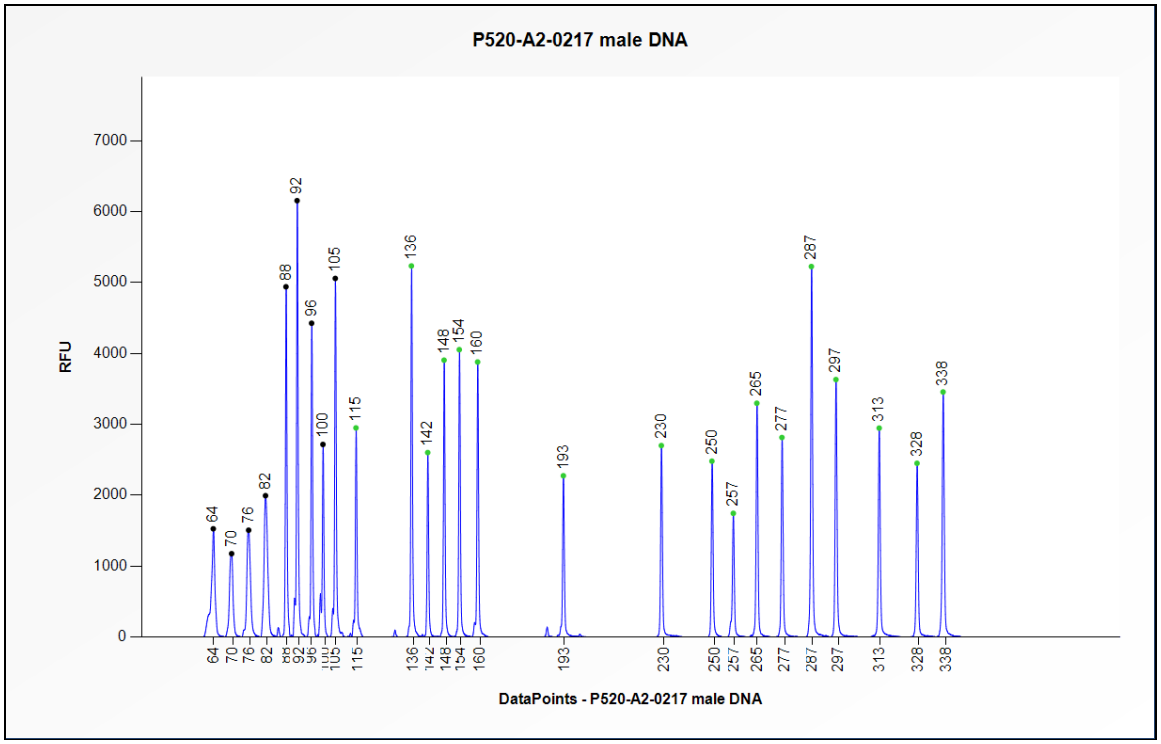


Figure 5. Capillary electrophoresis pattern from a sample of approximately 100 ng human male control DNA analysed with SALSA MLPA probemix P520-A2 MPN mix 2 (lot A2-0217).

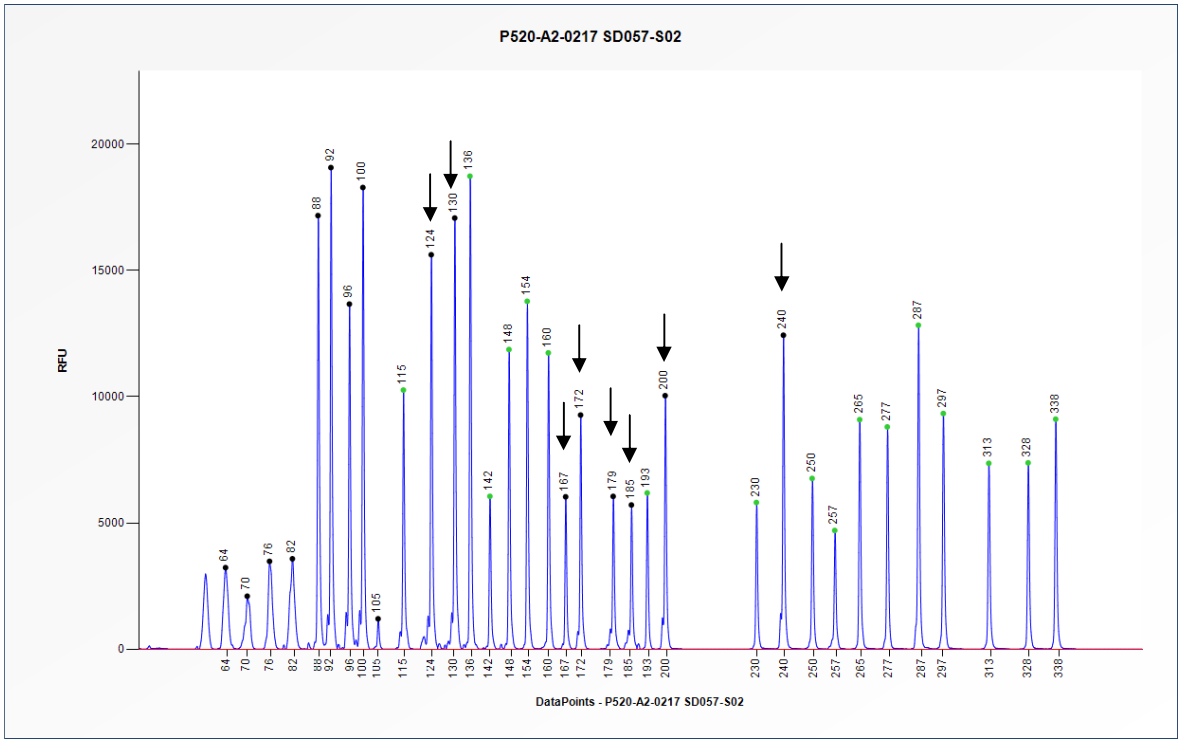


Figure 6. Capillary electrophoresis pattern of SD057-S02 sample DNA (approximately 100 ng) analysed with SALSA MLPA probemix P520-A2 MPN mix 2 (lot A2-0217). This sample DNA contains the sequences detected by the mutation-specific probes at 124 nt (CALR L367fs*46), 130 nt (CALR K385fs*47), 167 nt (JAK2 N542_E543del), 172 nt (JAK2 E543_D544del), 179 nt (MPL W515K), 185 nt (MPL W515L), 200 nt (KIT D816V) and 240 nt (JAK2 V617F) which are indicated with an arrow.

Please note the following: When probe signals at 167 nt and 172 nt both are present in the MLPA peak pattern, it is indicative for the JAK2 E543_D544del mutation. When only the probe signal at 167 nt is present, it is indicative for the JAK2 N542_E543del mutation.

Implemented Changes – compared to the previous product description version(s).

Version 09 – 26 August 2019 (T08)
- Information regarding JAK2 p.V617F WHO reference panel has been elaborated by including the proposed consensus values and a sample for 10% VAF on page 6.

Version 08 – 09 July 2019 (T08)
- Information regarding the estimated mutation burden of SD057 has been changed back to 1% upon the release of the improved SD057-S02.
- Sources for commercial positive reference samples updated on page 6.
- New picture added to page 10 on the CE pattern of SD057-S02 using P520-A2-0217.

Version 07 – 18 October 2018 (T08)
- Information regarding the estimated mutation burden of SD057 has been changed from 1% to 0.5-1%.
- Product description modified so that P520 probemix with SD057 should be used only as a qualitative tool (mutation present or absent) and not as a quantitative tool for measuring exact mutation burden.
- Remark added that internal or external reference standards should be included both in the validation phase and in experiments.
- Information added on page 3 about possibility of detecting mutations at low level in general population.
- Recommendation about confirmation of positive mutation calls with low mutation burden and possible methods used for this purpose added on page 5.
- Information regarding positive reference samples added and adjusted on page 6.
- Various minor textual changes throughout the document.

Version 06 – 27 September 2017 (T08)
- Small changes of probe lengths in Table 1, Table 2, Figure 5 and Figure 6 in order to better reflect the true lengths of the amplification products.
- Information on related SALSA® MLPA® probemixes updated.

Version 05 – 29 June 2017 (T08)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new pictures included).
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.
- Various textual changes throughout the document.

Version 04 – 14 December 2015 (T07)
- Product description adapted to new Sample DNA (Sample DNA information changed, new pictures included). SD057 is the new Sample DNA for P520-A1 probemix and replaces SD047 and SD048.

Version 03 – 13 July 2015 (T07)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included).