Product Description

SALSA® MLPA® Probemix P520-A2 MPN mix 2

To be used with the MLPA General Protocol.

Version A2
For complete product history see page 12.

The P520 MPN mix 2 assay allows mutations detection with high sensitivity. Only 1% allele burden is required for detection of the eight different mutations frequently found in myeloproliferative neoplasms (MPNs).

With each P520 MPN mix 2 order, an artificial DNA sample (SD057) with an estimated 1% allele burden is supplied for data binning in the fragment analysis procedure, and for serving as an artificial positive control for all the mutation-specific probes. This SD057 should be used in each P520 MPN mix 2 experiment for correct fragment binning and mutation calling.

Please note that this probemix should be used only as a tool for determining mutation presence (or absence) and NOT used as a quantitative tool for measuring exact mutation burden. In addition, positive controls should be included both in the validation phase and in experiments.

Catalogue numbers:

- **P520-025R:** SALSA MLPA Probemix P520 MPN mix 2, 25 reactions.
- **P520-050R:** SALSA MLPA Probemix P520 MPN mix 2, 50 reactions.
- **P520-100R:** SALSA MLPA Probemix P520 MPN mix 2, 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.

Note 1: The use of this product requires two modifications to the MLPA General Protocol. First, the PCR reaction should be prolonged from 35 to 40 cycles. Second, we strongly recommend to use ≥100 ng of DNA sample per MLPA reaction (maximum 400 ng of DNA) for most optimal and robust mutation detection with the P520 MPN mix 2.

P520 MPN mix 2 users 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 mutation calling.

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

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 *JAK2, MPL, CALR* and *KIT* 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 test sample, we recommend to perform sequencing of the *JAK2, MPL*, *CALR* and *KIT* genes to detect the rare or unknown mutations possibly present in a test sample.

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.

General information
The SALSA MLPA Probemix P520 MPN mix 2 is a **research use only (RUO)** assay for detection of eight different mutations frequently found in MPNs in *JAK2, MPL, CALR* and *KIT* genes.

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* p.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 p.V617F negative PV patients. P520-A2 MPN mix 2 contains three mutation-specific *JAK2* probes: one probe for p.V617F and two probes for the most common exon 12 mutations p.N542_E543del and p.E543_D544del.

Mutations in the *MPL* gene (1p34.2) are found in 4-11% of *JAK2* p.V617F negative ET and PMF patients. This problemix contains two mutation-specific probes for *MPL*, p.W515K and p.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 et al. 2013, Nangalia et al. 2013). *CALR* mutation-specific probes for the 52-bp deletion (p.L367fs*46, type 1) and 5-bp insertion (p.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.

This SALSA MLPA probemix is not CE/FDA registered for use in diagnostic procedures. Purchase of this product includes a limited license for research purposes.

Gene structure and transcript variants:
Entrez Gene shows transcript variants of each gene: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
For NM_ mRNA reference sequences: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Exon numbering
The *JAK2, MPL, CALR* and *KIT* exon numbering used in this P520-A2 MPN mix 2 product description is the exon numbering from the LRG_612 (*JAK2*), LRG_510 (*MPL*), LRG_828 (*CALR*) and LRG_307 (*KIT*). The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. As changes to the databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date.

Probemix content
The SALSA MLPA Probemix P520-A2 MPN mix 2 contains 25 MLPA probes with amplification products between 115 and 338 nucleotides (nt). This probemix contains eight probes specific for the *JAK2, MPL, CALR*

and *KIT* mutations which will only generate a signal when the mutation is present (see details of these mutations detected below).

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

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

Finally, 17 reference probes are included in P520-A2 probemix, detecting different autosomal chromosomal locations which are relatively stable in MPNs. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

In mutation-negative samples, only the 17 reference probes are expected to generate a signal. The remaining eight probes will only generate a signal when the corresponding mutation is present in the test sample. Please note that, however, mutation-specific probes can generate a low background signal in normal samples.

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, and specifically for this probemix in Figure 1 of this product description.

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).

Modifications to the MLPA General Protocol (One-Tube)

Please note that P520 MPN mix 2 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. **P520 MPN mix 2 users 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 mutation calling.** The standard One-Tube MLPA protocol should be followed, but using the following thermocycler program for the MLPA reaction:

DNA denaturation			
1.	98°C	5 minutes	
2.	25°C	pause	
Hybridisation reaction			
3.	95°C	1 minute	
4.	60°C	16-20 hours	
Ligation reaction			
5.	54°C	Pause	
6.	54°C	15 minutes	
7.	98°C	5 minutes	
8.	20°C	pause	
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	

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 ≤0.20 for all reference probes over the experiment.

Required specimens
Extracted DNA free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

Reference samples
A sufficient number (≥3) of reference samples should be included in each MLPA experiment for data normalisation. All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. Reference samples should be derived from different healthy individuals without a history of myeloproliferative neoplasms. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol (www.mrcholland.com).

Positive and negative control DNA samples
MRC Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. The quality of cell lines can change; therefore samples should be validated before use. Please find below some 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) – www.accuref.com (AccuRef).

We highly recommend inclusion 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.

Additionally, we highly recommend to also include **mutation positive samples (cell line or patient derived) extracted with the same DNA extraction method as the test samples** in MLPA experiments to optimise mutation calling for the test samples.

SALSA Threshold & Binning DNA SD057

The SD057 Threshold & Binning DNA provided with this probemix can aid the binning and serve as a mutation calling threshold sample for the eight mutation-specific probes: *CALR* p.L367fs*46 (S0999-L26702; 124 nt), *CALR* p.K385fs*47 (S1001-L26517; 130 nt), *JAK2* p.N542_E543del (16924-L21237; 167 nt), *JAK2* p.E543_D544del (16924-L21238; 172 nt), *MPL* p.W515K (S1048-SP0405-L29870; 181 nt), *MPL* p.W515L (S1048-SP0405-L29871; 186 nt), *KIT* p.D816V (17722-SP0542-L23707; 200 nt) and *JAK2* p.V617F (13190-L21572; 240 nt). SD057 Threshold & Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequence detected by the above mentioned probe. **NOTE that this SD057 sample DNA contains an estimated 1% burden of the above mentioned mutation-specific sequences**. Inclusion of one reaction with 5 µl SD057 (20 ng/µl) Threshold & Binning DNA with total of 100 ng of DNA in each MLPA experiment is essential 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. For reliable threshold determination and subsequently mutation calling, it is highly recommended to include three reactions of SD057 in each MLPA experiment (please see *Interpretation of results for mutation calls* section for more information). Furthermore, SD057 should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). It is strongly advised that all samples tested are extracted with the same method and derived from the same source of tissue. For further details, please consult the SD057 Threshold & Binning DNA product description, available online: www.mrcholland.com. **This product is for research use only (RUO).**

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.

In contrast to conventional MLPA probemixes, the Q-fragments in P520 MPN mix 2 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.

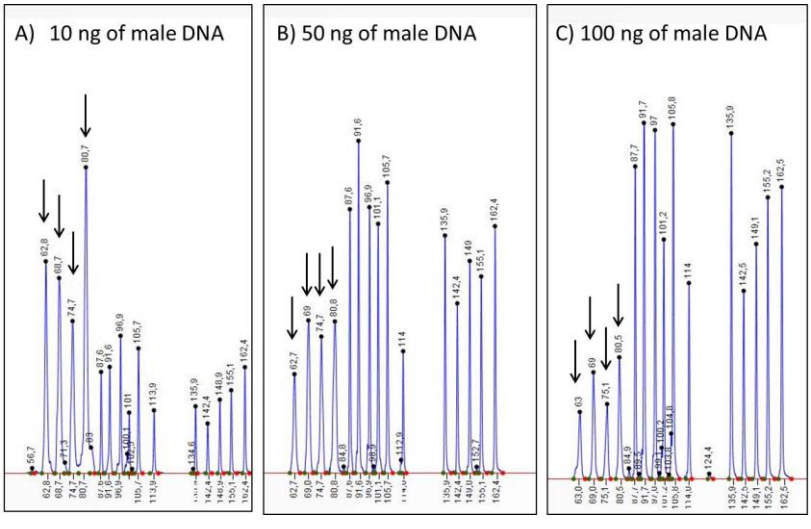

Figure 1. Q-fragments in the P520-A2 probemix. P520-A2 probemix analysed with A) 10 ng of male DNA; median of Q-fragments is higher than the 92 nt control fragment and in Coffalyser.Net analysis a red warning is indicated; B) 50 ng of male DNA; median of Q-fragments is higher than 33% of the 92 nt control 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.

Interpretation of results for mutation calls

The standard deviation of each individual reference probe over all the reference samples should be ≤0.20. Please note that Coffalyser.Net uses stricter cut-off for standard deviation, which is not applicable for this probemix with the condition that the experiment includes ≥3 reference samples extracted from the same tissue/sample type with same pre-analytical conditions and DNA extraction method. When these criteria are fulfilled, the assay can yield reliable results for point mutation detection and calling.

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 Threshold & Binning DNA serves as a threshold for the presence for the corresponding mutation. SD057 contains an estimated 1% allele burden for each aforementioned mutation. Given that **three reactions with SD057 are included in the experiment and defined as 'Positive reference' as 'sample type'** in *fragment analysis* tab, the intra ratio percentages in the Coffalyser 'comparative analysis experiment explorer' in *ratio overview* (for all samples) or *sample report* (per sample) tabs can be directly compared between SD057 for reliable mutation calling.

In case the signal of the mutation-specific probe in a sample of interest is higher than the average signal of the corresponding probe in the three SD057 reactions, serving as threshold samples, 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 average signal in the three SD057 reactions, this is regarded as a negative call (see examples in Figure 2).

When probe signals at 167 nt and 172 nt are both present in the MLPA peak pattern, and higher than in the SD057 threshold sample, it is indicative for the *JAK2* p.E543_D544del mutation. When only the probe signal at 167 nt is present, and higher than in the SD057 threshold sample, it is indicative for the *JAK2* p.N542_E543del mutation.

In addition to Coffalyser.Net analysis, visual examination of the P520 MPN mix 2 MLPA peak patterns is always necessary for mutation calling (in addition to Figure 2 for negative mutation calling, see also Figure 4 for saturated mutation-specific MLPA peaks) to confirm that the signals seen in the sample of interest are higher than in the SD057 threshold sample(s).

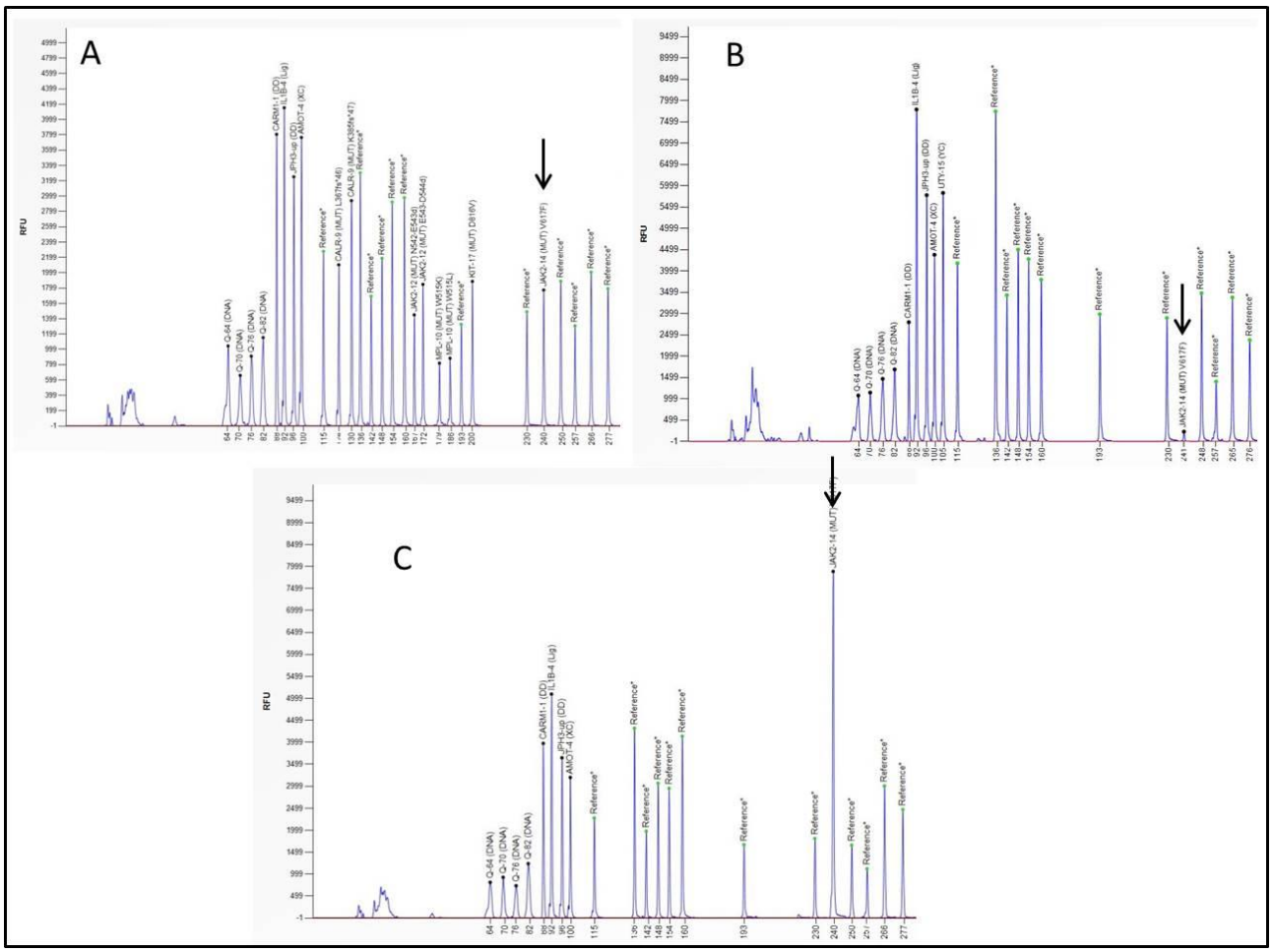

Figure 2. Mutation calling with the P520 MPN mix 2: examples of negative and positive mutation calls for the *JAK2* p.V617F point mutation. A) P520 MPN mix 2 analysed with SD057 Threshold & Binning DNA that contains an estimated 1% burden of the *JAK2* p.V617F mutation sequence. The peak of the *JAK2*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

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

When a test sample has >5% allele burden, the corresponding MLPA probe peak may become saturated in the MLPA peak pattern (see Figure 3 for further information). The saturated peak height is usually much higher than the upper threshold for relative fluorescence detection limit of capillary electrophoresis instrument.

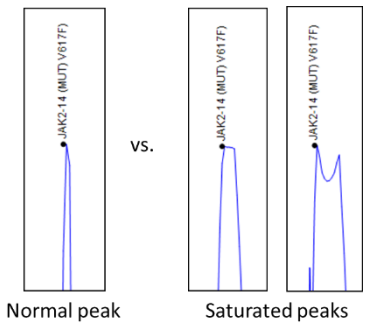

Figure 3. Normal vs. saturated MLPA peaks. Saturated peaks can be detected in the MLPA peak pattern by zooming in: a blunt end of an MLPA peak or alternatively a double peak at the very tip of the MLPA peak is then detected.

High allele burden (>5%) commonly results in an extra "shadow peak" ~15 nt before the real mutation-specific peak. This "shadow peak" can occur to any of the mutation-specific probes and it 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 in formation of the "shadow peak" (see Figure 4 for further information).

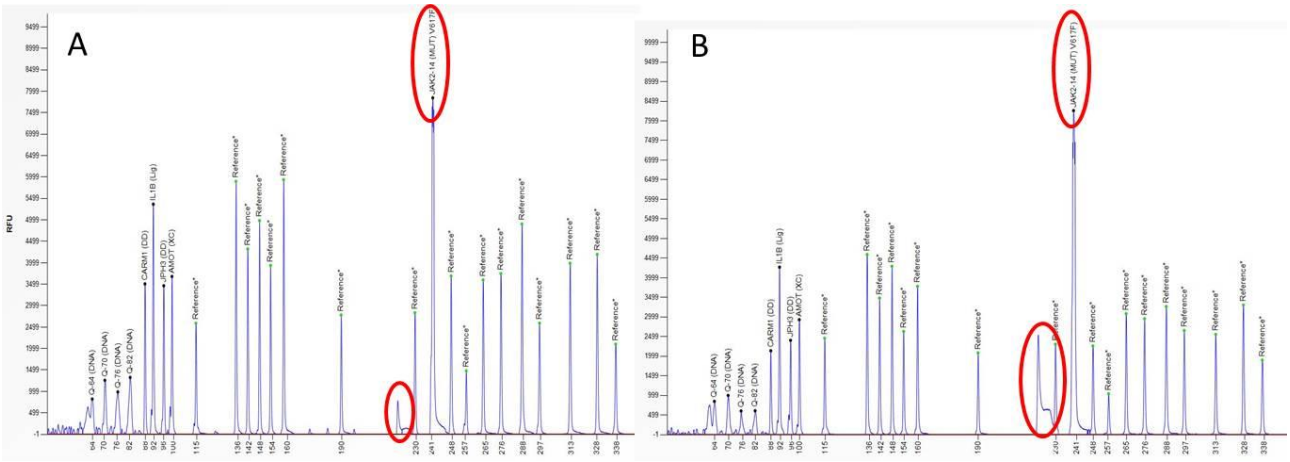

Figure 4. Saturated mutation-specific peaks in the MLPA peak pattern. A) As an example *JAK2* p.V617F positive test sample with 10% allele burden was analysed with the P520 MPN mix 2 and run at an ABI sequencer. The *JAK2* p.V617F specific peak at 240 nt is saturated and there is a "shadow peak" visible at 222 nt. B) The same *JAK2* p.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". Please note that with this dilution the other probe signals in the MLPA peak pattern are diminished as well.

Please note that the final ratios and mutation calling are affected both by percentage of tumour cells and by possible subclonality.

- False positive results: Literature suggests that some individuals in the general population can have *JAK2* p.V617F mutation-positive clones at low levels (See e.g. Sidon et al. 2006, Nielsen et al. 2013). Please note also that abnormalities detected by a single probe still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- False positive results with *CALR* p.L367fs*46=c.1092_1143del52 probe: We have received reports of experiments with a non-specific peak in all or some of the MLPA reactions. This peak was very close (in most cases about 1 nt longer) to the peak expected for the *CALR* p.L367fs*46 mutation-specific probe. Even when SD057 samples were used for binning, this non-specific peak was assigned to *CALR* p.L367fs*46 probe by Coffalyser.Net and thus it yielded a false-positive result in most severe cases. The formation of this non-specific peak is caused by not strictly following the instructions of the MLPA protocol (especially when causing a delay in continuing the thermocycler program after adding the PCR master mix). Low level signals comparable or lower to the reference probes (with +1nt peak when compared to SD057 mutation-specific peak) obtained with this *CALR* mutation probe should therefore be treated with caution. For more information on this issue, please contact info@mrcholland.com.
- 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. The risk of 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 might 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.

Limitations of the procedure

- This SALSA MLPA Probemix P520 MPN mix 2 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, and this can lead to false positive or negative results! In addition, some probe signals can be more sensitive to sample purity and small changes in experimental conditions. Unlike the majority of MLPA probemixes, this P520 MPN mix 2 does not provide copy number information of the target probes.
- MLPA cannot detect mutations that are not included in this assay. 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.
- MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Point mutations may not be detected if the percentage of tumour cells is low. In addition, subclonality of the mutations 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 test sample, especially in tumour samples with more chaotic karyotypes.

Confirmation of results

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.

We recommended to use P420 MPN mix 1 for test samples when there is a saturated mutation-specific signal in P520 MPN mix 2 in case of >10% allele burden present in the test sample and in order to confirm the result. The P420 MPN mix 1 assay is an 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 MPN mix 2 as qualitative tool for mutation detection.

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 or false negative results due to SNVs and unusual results to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P520-A2 MPN mix 2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	Mutation
64-105	Control fragments – see table in probemix content section for more information		
115	Reference probe S0973-L26704	4p13	
124 § +	**CALR probe** S0999-L26702		**p.L367fs*46**=c.1092_1143del52
130 § ×	**CALR probe** S1001-L26517		**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		**p.N542_E543del**=c.1624_1629delAATGAA
172 § £	**JAK2 probe** 16924-L21238		**p.E543_D544del**=c.1627_1632delGAAGAT
181 § Ж	**MPL probe** S1048-SP0405-L29870		**p.W515K**=c.1543_1544TG>AA
186 § Ж	**MPL probe** S1048-SP0405-L29871		**p.W515L**=c.1544G>T
193 ¥	Reference probe 11556-L26606	5q31	
200 § Ж	**KIT probe** 17722-SP0542-L23707		**p.D816V**=c.2447A>T
230	Reference probe 17130-L26574	11p11	
240 § ∑	**JAK2 probe** 13190-L21572		**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	2q13	

[a] See section Exon numbering on page 2 for more information.

§ Mutation-specific probe. This probe will generate a signal when the mutation is present.

+ *CALR* p.L367fs*46 =c.1092_1143del52 probe is sensitive to any deviation from the adjusted MLPA protocol (40 cycles, see page 3-4). Unspecific low level signals comparable or lower to the reference probes (with ~+1 nt peak length when compared to SD057) obtained with this *CALR* mutation-specific probe should therefore be treated with caution. For more information contact info@mrcholland.com.

× *CALR* p.K385fs*47=c.1154_1155insTTGTC probe has been reported to have unspecific low level background signals (with ~-2 nt peak length when compared to SD057). Low level signals obtained with this *CALR* mutation probe should therefore be treated with caution.

Ж This probe consists of three parts and has two ligation sites.

£ **When probe signals at 167 nt and 172 nt are both present in the MLPA peak pattern, and the signals are higher than in the SD057 threshold sample, it is indicative for the *JAK2* p.E543_D544del mutation. When only the probe signal at 167 nt is present, and the signal is higher than in the SD057 threshold sample, it is indicative for the *JAK2* p.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* p.V617F samples with an 1% allele burden in the internal quality tests at MRC Holland. See Figure 2 for further information on this.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Table 2. P520-A2 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene / exon[a] / Mutation	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)
***MPL* gene**, at 1p34.2, indicated ligation sites are in NM_005373.3. Mutations in the thrombopoietin receptor (*MPL*) gene, most commonly p.W515L and p.W515K substitutions in exon 10, are detected in patients with ET and PMF, which can support diagnosis when *JAK2* is not mutated. p.W515L and p.W515K are reported to be the predominant *MPL* mutations in MPNs (Ma W et al. 2011).				
186 § Ж	S1048-SP0405-L29871	***MPL***, exon 10, **p.W515L=c.1544G>T**	1575-1576 and 25 nt after exon 10	GCTGCTGATGT**T**-46nt spanning oligo-TGGCGGTGGACC
181 § Ж	S1048-SP0405-L29870	***MPL***, exon 10, **p.W515K=c.1543_1544TG>AA**	1575-1576 and 25 nt after exon 10	GCTGCTGAGG**AA**-46nt spanning oligo-TGGCGGTGGACC
***KIT* gene**, at 4q12, indicated ligation sites are in NM_000222.3. Somatic activating *KIT* p.D816V mutation is present in more than 97% of SM patient samples (Erben P et al. 2014). This p.D816V mutation results in ligand-independent activation of c-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**	2505-2504 and 2467-2466 *reverse*	CATTCTTGATG**A**-38nt spanning oligo-CCATGAGTAAGG
***JAK2* gene,** at 9q24.1, indicated ligation sites are in NM_004972.4. 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* p.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). The most common *JAK2* mutations in exon 12 are the following 6-bp deletions: p.N542_E543del and p.E543_D544del.				
167 § £	16924-L21237	***JAK2*,** exon 12, **p.N542_E543del** **=c.1624_1629delAATGAA**	2099-2100	AAAATCAGAGAT-TTGATATTTGTA
172 § £	16924-L21238	***JAK2*,** exon 12, **p.E543_D544del** **=c.1627_1632delGAAGAT**	2099-2100	AAAATCAGAAAT-TTGATATTTGTA
240 § ∑	13190-L21572	***JAK2*,** exon 14, **p.V617F=c.1849G>T**	2316-2315 *reverse*	GTCTCCACAGA**A**-ACATACTCCATA
***CALR* gene**, at 19p13.13, indicated ligation sites are in NM_004343.4. 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=p.L367fs*46) and 5-bp insertion (type 2=p.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, Nangalia J et al. 2013).				
130 § ×	S1001-L26517	***CALR***, exon 9, **p.K385fs*47** **=c.1154_1155insTTGTC**	1225-1226	CAGAGGACAA**TT**-**GTC**GGAGGATGA
124 § +	S0999-L26702	***CALR***, exon 9, **p.L367fs*46** **=c.1092_1143del52**	1163-1214 *reverse*	CTTGTCCTCTGC-TCCTCGTCCTGT

[a] See section Exon numbering on page 2 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.

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SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Related SALSA MLPA probemixes

For related products, see the product page on our website.

References

- Erben P et al. (2014). The KIT D816V expressed allele burden for diagnosis and disease monitoring of systemic mastocytosis. *Ann Hematol.* 93:81-8.
- Hömig-Hölzel C and Savola S. (2012). Multiplex ligation-dependent probe amplification (MLPA) in tumor diagnostics and prognostics. *Diagn Mol Pathol*. 21:189-206.
- Klampfl T et al. (2013). Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med*. 369:2379-90.
- Ma W et al. (2011). MPL mutation profile in JAK2 mutation-negative patients with myeloproliferative disorders. *Diagn Mol Pathol.* 20:34-9.
- Nangalia J et al. (2013). Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med*. 369:2391-405.
- Nielsen C et al. (2013). Diagnostic value of JAK2 V617F somatic mutation for myeloproliferative cancer in 49 488 individuals from the general population. *Br J Haematol*. 160:70-9.
- 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.
- Sidon P et al. (2006). The JAK2 V617F mutation is detectable at very low level in peripheral blood of healthy donors. *Leukemia*. 20:1622.
- 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.
- Tefferi A (2010). Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. *Leukemia.* 24:1128–38.

P520 product history	
Version	*Modification*
A2	Several probes have been changed in length no change in the sequence detected.
A1	First release.

Implemented changes in the product description
Version A2-07 – 20 May 2025 (04P) - Adjusted SD057 name to SALSA Threshold & Binning DNA throughout document. - Related SALSA MLPA products section replaced with reference to product page on website. Version A2-06 – 18 June 2024 (04P) - Highlighted on page 1 and 3 that P520 MPN mix2 users 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 mutation calling. - Added a text about false positive results with *CALR* p.L367fs*46 =c.1092_1143del52 probe on page 8. - Added a warning for low level unspecific signal for both *CALR* p.L367fs*46 =c.1092_1143del52 (at 124 nt) and *CALR* p.K385fs*47=c.1154_1155insTTGTC (at 130 nt) probes in Tables 1 and 2. - Minor typos and adjustments throughout the document. Version A2-05 – 03 May 2023 (04P) - Added a sentence on page 6 that Coffalyser.net uses stricter cut-off for standard deviation, which is not applicable for this probemix when the experiment includes ≥3 reference samples. Version A2-04 – 23 March 2023 (04P) - Correction for the link in included on page 4. - Added in SD057 information that for threshold purposes for reliable mutation detection and calling, it is highly recommended to include three reactions of SD057 in each MLPA experiment. - Various clarifications in the *Interpretation of results for mutation calls* -section on pages 6, including Coffalyser analysis instructions for mutation calling. - Minor clarifications and adjustments throughout the document. Version A2-03 – 13 July 2022 (04P) - Text about SD057 on page 5 is corrected: SD057 should be used in each P520 experiment, not only for the initial experiments.

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