Instructions for Use
SALSA® MLPA® Probemix P335 ALL-IKZF1

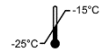
See also the MLPA General Protocol, the product description of the SALSA® MLPA® Reagent Kit, and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix P335 ALL-IKZF1 product page on our website to find Certificates of Analysis and a list of related products.

Product name	**SALSA® MLPA® Probemix P335 ALL-IKZF1**
Version	**C2**
Catalogue numbers	**P335-025R (25 reactions)** **P335-050R (50 reactions)** **P335-100R (100 reactions)**
Basic UDI-DI:	**n.a.**
Ingredients	**Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA**

Additional Test Components	**Catalogue numbers**
SALSA® MLPA® Reagent Kit	EK1-FAM EK1-CY5 EK5-FAM EK5-CY5 EK20-FAM

Storage and Shelf Life

Recommended conditions		

A shelf life of until the expiry date is guaranteed, also after opening when stored in the original packaging under recommended conditions. For the exact expiry date, see the label on the vial. This product should not be exposed to more than 25 freeze-thaw cycles. Do not use the product if the packaging is damaged or opened. Leave chemicals in original containers. Waste material must be disposed of in accordance with the national and local regulations.

Regulatory Status	
IVD	EUROPE CE COLOMBIA ISRAEL COSTA RICA
RUO	ALL OTHER COUNTRIES

Label Symbols			
IVD	In Vitro Diagnostic	RUO	Research Use Only

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Any serious incident that has occurred in relation to this product should be reported to MRC Holland and the competent authority of the Member State in which the user and/or the patient is located.

Changes in this Product Version:
As compared to version C1, lengths of several probes are adjusted, but no changes in the sequences detected.

1. Intended Purpose

The SALSA MLPA Probemix P335 ALL-IKZF1 is an in vitro diagnostic (IVD)[1] or a research use only (RUO) semi-quantitative manual assay[2] for the detection of deletions of the *IKZF1* gene for stratification of patients with acute lymphoblastic leukemia (ALL) into prognostic subgroups[3]. The SALSA MLPA Probemix P335 ALL-IKZF1 is a RUO assay[2] for the detection of deletions or duplications in B-cell differentiation and cell cycle control genes (*EBF1*, *CDKN2A/2B*, *PAX5*, *ETV6*, *BTG1* and *RB1*) and in the PAR1 region. This assay is for use on genomic DNA isolated from human peripheral whole blood and bone marrow specimens.

Copy number alterations (CNAs) detected with P335 ALL-IKZF1 should be confirmed with a different technique. In particular, CNAs detected by only a single probe always require confirmation by another method. In the majority of patients, defects in the *IKZF1* gene are deletions, but point mutations can occur which will not be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis of the *IKZF1* gene.

Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes or population screening.

[1] Please note that this probemix is for in vitro diagnostic (IVD) use in the countries specified on page 1 of this product description. In all other countries, the product is for research use only (RUO).

[2] To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

[3] Certain probes targeting additional genes included in P335 ALL-IKZF1 may only be used in a research setting. The following table summarises which probes are for IVD use or exclusively restricted to be used in a research setting:

IVD Target	RUO Targets
IKZF1	*EBF1*, *CDKN2A/2B*, *PAX5*, *ETV6*, *BTG1*, *RB1*, and the PAR1 region

2. Sample Requirements

Specimen	50-250 ng purified human genomic DNA, free from heparin, dissolved in 5 µl $TE_{0.1}$ buffer, pH 8.0-8.5
Collection Method	Standard methods
Extraction Method	Methods tested by MRC Holland: • QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual) • Promega Wizard Genomic DNA Purification Kit (manual) • Salting out (manual)

Sample Types		
Test Sample	• Provided by user • ≥30% tumour cells (recommended: ≥50%)	
Reference Samples (Required)	• Provided by user • Extraction method, tissue type, DNA concentration and treatment as similar as possible in all test and reference samples. • Have a normal copy number and ≤0.10 standard deviation for all probes. • At least three* independent reference samples required in each experiment for proper data normalisation. Derived from unrelated male individuals from families without a history of ALL. • Should be from healthy male individuals to facilitate interpretation (see appendix for more information).	
No-DNA Control (Preferably)	• Provided by user • $TE_{0.1}$ buffer instead of DNA • To check for DNA contamination	
Positive Control Samples (Preferably)	• Provided by user if applicable, or	
	Available from third parties	See the table of positive samples on the probemix product page on our website.
Validation Samples (Required)	• In the validation experiments of this probemix, DNA samples from healthy male individuals should be used.	

*When testing >21 samples, include one extra reference for each 7 test sample.

3. Test Procedure

See the MLPA General Protocol.

4. Quality Control, Data Analysis, and Troubleshooting

Quality Control Fragments in the Probemix	
Length (nt)	**Function**
64-70-76-82	DNA quantity control fragments
88-96	DNA denaturation control fragments
92	Benchmark fragment
100	Chromosome X presence control fragment
105	Chromosome Y presence control fragment

Coffalyser.Net should be used for data analysis in combination with the appropriate product and lot-specific Coffalyser sheet. See the Coffalyser.Net Reference Manual for details on data analysis and quality control.

For troubleshooting help, see the additional resources offered on our support portal.

5. Interpretation of Results

Determining Typical Values in Normal and Affected Populations

The typical final ratio (FR) values stated in the copy number tables were determined in a validation study with samples containing abnormal copy numbers. The standard deviation of each individual probe over all the reference samples was ≤0.10.

Expected Results of Reference Probes

Final Ratio (FR)	Copy Number	Description
0.80 – 1.20	2	Normal

Typical Results of Probes Targeting Two Copies (*IKZF1*, *EBF1*, *CDKN2A/2B*, *PAX5*, *ETV6*, *BTG1*, *RB1*, and the PAR1 region)

Final Ratio (FR)	Copy Number	Description
0	0	Homozygous deletion
0.40 – 0.65	1	Heterozygous deletion
0.80 – 1.20	**2**	**Normal**
1.30 – 1.65	3	Heterozygous gain
1.75 – 2.15	4	Homozygous gain or Heterozygous triplication
All other values	-	Ambiguous

The tables illustrate the relationship between final probe ratio and corresponding copy number. Test results are expected to center around these values. Ambiguous values can indicate a technical problem, but may also reflect a biological cause.

Final ratios are affected both by percentage of tumour cells and by possible subclonality. It is important to use Coffalyser.Net to determine the significance of values found.

For a detailed interpretation guide, see the Appendix.

6. Performance Characteristics

IKZF1 deletions can be identified in approximately 70% of children with *BCR-ABL1* positive (Philadelphia chromosome, Ph+) ALL (2% of all pediatric ALL cases), in 10-15% of Philadelphia chromosome negative (Ph−) pediatric ALL cases and in 40% of adult B-ALL cases (Bernt and Hunger 2014; Lejman et al. 2022; van der Sligte et al. 2015).

Genomic deletions in *IKZF1* are either whole gene deletions (25-50% of all *IKZF1* deletions; Mullighan et al. 2009; Palmi et al. 2013) or intragenic deletions, with the most frequent intragenic deletion (exons 4-7) comprising 30-55% of all deletions (Kastner et al. 2013, Iacobucci et al. 2009). The analytical sensitivity and specificity for the detection of deletions or duplications in the *IKZF1* gene is very high and can be considered >99%.

Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

Content – Probe Details Sorted by Chromosomal Position

Chr. position	Target	Exon	Distance to next probe	Length (nt)	Probe number	Warnings
5q33.3	*EBF1*	Exon 16	14.0 kb	463	23047-L32673	¥
5q33.3	*EBF1*	Exon 14	65.2 kb	436	13868-L22053	
5q33.3	*EBF1*	Exon 10	322.2 kb	372	14059-L30509	
5q33.3	*EBF1*	Exon 1		226	12509-L13559	
7p12.2	*IKZF1*	Exon 1	14.2 kb	269	13877-L15918	«
7p12.2	*IKZF1*	Exon 2	8.6 kb	208	14056-L15654	«
7p12.2	*IKZF1*	Exon 3	74.0 kb	379	15427-L22113	
7p12.2	*IKZF1*	Exon 4	5.9 kb	264	13873-L15917	
7p12.2	*IKZF1*	Exon 5	4.8 kb	142	21511-L30001	
7p12.2	*IKZF1*	Exon 6	4.4 kb	470	14061-L22112	
7p12.2	*IKZF1*	Exon 7	9.3 kb	344	13869-L32515	¥
7p12.2	*IKZF1*	Exon 8		288	17109-L20256	
9p24.1	*JAK2*		17 **M**b	157	07452-L32509	¥ ¬
9p21.3	*CDKN2A*	Exon 3	7.1 kb	309	17814-L22631	
9p21.3	*CDKN2A*	Upstream [Intron 1] (Exon 1)	30.9 kb	252	10333-L30127	Ø ₱
9p21.3	*CDKN2B*	Exon 2	15 **M**b	239	16059-L30167	
9p13.2	*PAX5*	Exon 10	42.0 kb	394	17842-L22633	
9p13.2	*PAX5*	Exon 8	41.4 kb	274	17840-L22037	
9p13.2	*PAX5*	Exon 7	43.2 kb	337	17841-L22038	
9p13.2	*PAX5*	Exon 6	36.1 kb	202	17839-L22036	
9p13.2	*PAX5*	Exon 5	18.0 kb	282	13870-L15920	«
9p13.2	*PAX5*	Exon 2	13.6 kb	172	14647-L15394	
9p13.2	*PAX5*	Exon 1		161	12501-L32513	¥ +
12p13.2	*ETV6*	Exon 1	0.1 kb	301	14058-L15656	«
12p13.2	*ETV6*	Exon 1	102.4 kb	389	14060-L32514	¥ «
12p13.2	*ETV6*	Exon 2	86.6 kb	401	13875-L22014	
12p13.2	*ETV6*	Exon 3	30.3 kb	244	13874-L17160	
12p13.2	*ETV6*	Exon 5	22.3 kb	485	13871-L22009	
12p13.2	*ETV6*	Exon 8	79 **M**b	196	17838-L22035	
12q21.33	*BTG1*-area		348.3 kb	178	18021-L22630	¬
12q21.33	*BTG1*-area		55.7 kb	409	18022-L22363	¬
12q21.33	*BTG1*	Exon 2	1.6 kb	330	12553-L22632	#
12q21.33	*BTG1*	Exon 1		232	21378-L30126	
13q14.2	*RB1*	Exon 6	30.2 kb	220	01782-L01346	+
13q14.2	*RB1*	Intron 13 (Exon 14)	76.9 kb	315	01789-L22025	Ø
13q14.2	*RB1*	Exon 19	16.8 kb	358	01792-L22295	
13q14.2	*RB1*	Intron 23 (Exon 24)	3.8 kb	427	01797-L16909	Ø
13q14.2	*RB1*	Exon 26		445	01799-L01362	
Xp22.33/ Yp11.32	*SHOX*-area (PAR1)		510.7 kb	149	05648-L32510	¥ ∫
Xp22.33/ Yp11.32	*CRLF2* (PAR1)		93.0 kb	136	13889-L15427	∫
Xp22.33/ Yp11.32	*CSF2RA* (PAR1)		41.4 kb	166	13892-L16221	∫
Xp22.33/ Yp11.32	*IL3RA* (PAR1)		129.4 kb	351	13907-L22294	∫
Xp22.33/ Yp11.32	*P2RY8* (PAR1)		1.3 **M**b	184	17837-L15740	∫
Yp11.31	*ZFY*			120	S0135-L27684	¬
1p21	Reference - *COL11A1*			214	13265-L32718	¥
2p15	Reference - *PEX13*			504	09870-L19465	
2q24	Reference - *SCN1A*			258	04534-L22019	
3p25	Reference - *BRK1*			364	14675-L16327	
3p12	Reference - *GBE1*			494	15203-L16978	
3q11	Reference - *CPOX*			478	14846-L22111	
5p15	Reference - *SEMA5A*			454	18691-L02476	
7q11	Reference - *POR*			124	15370-L13762	
8q22	Reference - *VPS13B*			418	10063-L30260	
9q34	Reference - *COL5A1*			295	10435-L22110	Ψ
11q12	Reference - *BEST1*			192	06941-L32511	¥
15q21	Reference - *FBN1*			324	03918-L20270	
16p13	Reference - *ABAT*			130	13867-L15385	

Probe lengths may vary slightly depending on capillary electrophoresis instrument settings. Please see the most up to date Coffalyser sheet for exact probe lengths obtained at MRC Holland.
The *EBF1*, *IKZF1*, *CDKN2A*, *CDKN2B*, *PAX5*, *ETV6*, *BTG1*, and *RB1* exon numbers are based on MANE Select transcripts. For more information, see the probe sequence document available on the product page at www.mrcholland.com. For *CDKN2A*, probe target exon number according to the MANE Clinical Plus transcript is given in square brackets when it differs from the MANE Select transcript. Annotations of several probes with targets at the edge of or slightly outside the coding region, were altered. The exon numbering from the previous version of this product description is disclosed between brackets.
Chromosomal bands are based on: hg18.

7. Precautions and Warnings

Probe changes

¥	Probe changed in this product version (C2). Minor alteration, no change in sequence detected.

Probe warnings

¬	These probes are flanking probe, included to help determine the extent of a deletion/duplication. Copy number alterations of flanking probes are unlikely to be related to the condition tested.
«	These probes are located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation.
Ø	These probes target a sequences outside of the known coding region.
#	The specificity of this probe relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.
+	The ligation site of these probes is >20 nt away from the nearest exon. For more information, download the probe sequence sheet from the probemix-specific page on www.mrcholland.com.
∫	These probes target the PAR1 region, which is present in two copies per cell in both XX females and XY males. Please note that a decrease in signal may also be due to a loss of the Y chromosome in male samples, and mosaic loss of the Y chromosome is a common feature in the blood of elderly men (Gutiérrez-Hurtado et al. 2024). Y chromosome loss can be detected by comparing the PAR1 results to the ZFY probe at 120 nt, and to the Y-specific control fragment at 105 nt. See appendix for more information.
Ψ	In comparison to focal chromosome 9p deletions, including the *JAK2*, *CDKN2A/2B* and *PAX5* genes, the *COL5A1* probe at 9q34.3 is also affected in case of complete chromosome 9 deletions.
₽	This probe targets a sequence 138 nt upstream of the *CDKN2A* MANE Select transcript (NM_000077.5, $P16^{INK4A}$), and in intron 1, 3.8 kb before exon 2, of the *CDKN2A* MANE Plus Clinical transcript (NM_058195.4, $p14^{ARF}$).

Probemix-specific precautions

1. This product is not known to contain any harmful agents. Based on the concentrations present, none of the ingredients are hazardous as defined by the Hazard Communication Standard. **A Safety Data Sheet (SDS) is not required for this product**: none of the ingredients contain dangerous substances at concentrations requiring distribution of an SDS (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and 1907/2006 [REACH] and amendments).
2. Sample or technical artefacts may appear as a (mosaic) copy number change of the whole/partial gene. Whole/partial gene deletions or duplications should therefore be confirmed by analysis of an independent DNA sample, to exclude false positive results.
3. Small changes (e.g. SNVs, small indels) in the sequence targeted by a probe can cause false positive results, even when >20 nt from the probe ligation site. Sequencing of the target region is recommended. Please contact MRC Holland for more information: info@mrcholland.com.
4. Copy number alterations of reference probes are unlikely to be related to the condition tested.

Technique-specific precautions
See the MLPA General Protocol.

8. Limitations

Probemix-specific limitations

1. The clinical significance of the following findings is not yet fully established: deletions and duplications in *EBF1*, *CDKN2A/2B*, *PAX5*, *ETV6*, *BTG1*, *RB1*, and the PAR1 region.
2. Duplication of the *IKZF1* gene is not expected in ALL patient samples, except in the context of larger duplications within or of chromosome arm 7p. Duplication of *IKZF1* is of unknown clinical significance.
3. MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA was extracted. Gains or losses of genomic regions or genes may not be detected if the percentage of tumour cells is low. The minimum percentage of tumour cells required for reliable analysis is 30% (Al Zaabi et al. 2010, Coll-Mulet et al. 2008). We advise using tumour samples with at least 50% tumour cell content. Therefore, tumour samples should be evaluated by a pathologist before the extraction of DNA. See appendix for more information. In addition, subclonality of the aberration affects the final ratio of the corresponding probe(s). Furthermore, there is always a possibility that one or more reference probes *do* show a copy number alteration in a patient sample.

Technique-specific limitations
See the MLPA General Protocol.

9. References Cited in this IFU

1. Al Zaabi EA et al. (2010). Multiplex ligation-dependent probe amplification versus multiprobe fluorescence in situ hybridization to detect genomic aberrations in chronic lymphocytic leukemia: a tertiary center experience. J Mol Diag. 12:197-203.
2. Bernt KM and Hunger SP (2014). Current concepts in pediatric Philadelphia chromosome-positive acute lymphoblastic leukemia. Front Oncol. 4:54.
3. Coll-Mulet L et al. (2008). Multiplex ligation-dependent probe amplification for detection of genomic alterations in chronic lymphocytic leukaemia. Br J Haematol. 142:793-801.
4. Gutiérrez-Hurtado IA et al. (2024) Loss of the Y Chromosome: A Review of Molecular Mechanisms, Age Inference, and Implications for Men's Health. *Int J Mol Sci.* 25(8):4230.
5. Iacobucci I et al. (2009). Identification and molecular characterization of recurrent genomic deletions on 7p12 in the IKZF1 gene in a large cohort of BCR-ABL1–positive acute lymphoblastic leukemia patients: on behalf of Gruppo Italiano Malattie Ematologiche dell'Adulto Acute Leukemia Working Party (GIMEMAAL WP). Blood. 114:2159-67.
6. Kastner P et al. (2013). Function of Ikaros as a tumor suppressor in B cell acute lymphoblastic leukemia. Am J Blood Res. 3:1-13.
7. Lejman M et al. (2022). Genetic biomarkers and their clinical implications in B-cell acute lymphoblastic leukemia in children. Int J Mol Sci. 23:2755.
8. Mullighan CG et al. (2009). Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med. 360: 470-80.

9. Palmi C et al. (2013). What is the relevance of Ikaros gene deletions as prognostic marker in pediatric Philadelphia negative B-cell precursor acute lymphoblastic leukemia? Haematologica. 98:1226-31.
10. Van der Sligte NE et al. (2015). Effect of IKZF1 deletions on signal transduction pathways in Philadelphia chromosome negative pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Exp Hematol Oncol. 4:23.

Implemented changes in the product description
Version C2-03 – 17 April 2025 (03S) - Product description adapted to a new template. - Product is now registered for IVD use in Colombia. - Product is no longer registered for IVD use in Morocco. - Description of probe targets at the edge of or slightly outside the coding region has been adjusted. No change in actual target sites. - Intended purpose was updated adjusting gene name formatting and specifying the assay is manual. - Warning added for probe 13870-L15920 being located in a GC-rich region. - SNV rs556167410 can affect the signal of probe 10435-L22110. However, the warning for this SNV present in previous product description versions has been replaced by a general warning for small sequence changes, included in section Precautions and Warnings. - Information about interpretation of PAR1 region probe signal was moved to a probe warning.

10. Appendix

Interpretation of results: male reference samples

In this probemix, the 120 nt Y-specific target probe (ZFY) is included as a flanking probe to help determine the extent of a deletion in the PAR1 region and to detect loss of the whole Y chromosome in male samples. To ensure that the comparative analysis can be completed for all samples, **only male reference samples must be used**. The comparative analysis will fail if female reference samples are used.

- In male samples, the 120 nt Y-specific target probe will be counted as an "additional" probe when using Coffalyser.Net software (see Figure 1). In healthy male individuals a final ratio of ~1 will be obtained for the Y-specific target probe after the comparative analysis. If the Y chromosome is lost in male samples, rather than deletion of the PAR1 region only, this can also be confirmed by visual examination of the 105 nt Y-fragment peak in the electropherogram (fragment analysis).
- In female samples, the 120 nt Y-specific target probe will be reported as "absent", i.e. will have a final ratio of 0, after the comparative analysis.

sample name	sample type	bin smpl	FRSS	FMRS	probes	DNA	DD	X	Y
Reference sample 1	reference	☐	▮▮▮	▮▮▮	✓ 57/57	✓	✓	✓	✓
Reference sample 2	reference	☐	▮▮▮	▮▮▮	✓ 57/57	✓	✓	✓	✓
Reference sample 3	reference	☐	▮▮▮	▮▮▮	✓ 57/57	✓	✓	✓	✓
Sample	sample	☐	▮▮▮	▮▮▮	✓ 56/56	✓	✓	✓	●
noDNA	no DNA	☐	▮▮▮		✓ 0/0	●	●	●	●

Figure 1. Example fragment analysis overview in Coffalyser.Net with three male reference samples, a female test sample and a no DNA reaction. Note that the number of detected/expected probes differs between male (57/57) and female (56/56) samples.

Interpretation of results: tumour cell percentage and subclonality

In case of a heterozygous deletion that is subclonal and/or the sample contains a lower percentage of tumour cells, the FR might not be in the expected range of 0.40-0.65 (as indicated in the table in section 4, above). For example, in case of ~50% tumour cell content or a copy number alteration present in ~50% of the tumour cells, the FR will be ~0.75. However, the same FR (0.75) will also be found for a sample with a tumour cell percentage of 25% or a subclone comprising 25% of all tumour cells that harbours a homozygous deletion. The MLPA technique cannot discriminate between these two scenarios.