

MR. THOMAS KRISTENSEN (Orcid ID : 0000-0002-9834-8771)

DR. SIGURD BROESBY-OLSEN (Orcid ID : 0000-0002-1558-8471)

Article type : Letter to the Editor

Title:

Clinical validation of a new commercial highly sensitive *KIT* D816V mutation analysis in mastocytosis

Authors

Thomas Kristensen, PhD¹

Hanne Vestergaard, MD, PhD²

Carsten Bindslev-Jensen, MD, PhD, DMSci^{3,4}

Michael Boe Møller, MD, DMSc¹

Sigurd Broesby-Olsen, MD^{3,4}

on behalf of the Mastocytosis Centre Odense University Hospital (MastOUH)

¹Department of Pathology, Odense University Hospital, Odense, Denmark

²Department of Hematology, Odense University Hospital, Odense, Denmark

³Department of Dermatology and Allergy Centre, Odense University Hospital, Odense, Denmark

⁴Odense Research Center for Anaphylaxis (ORCA), Odense University Hospital, Odense, Denmark

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14165](https://doi.org/10.1111/ALL.14165)

This article is protected by copyright. All rights reserved

Correspondence

Thomas Kristensen, Department of Pathology, Odense University Hospital, Winsloewparken 15, DK-5000 Odense C, Denmark. Phone (+45)65411660. E-mail: thomas.kielsgaard.kristensen@rsyd.dk

Funding

The PentaBase-assay reagents used in the present study was provided by PentaBase Aps.

Text word count: 1000

Number of figures: 2

Number of tables: 0

Number of references: 9

Supplementary data: Supplementary Table S1, Supplementary Table S2 and Supplementary Figure S1

To the Editor

The majority of adult mastocytosis patients carry the somatic *KIT* D816V mutation.¹ Recent advances in mutation analysis have facilitated increased use of *KIT* D816V as diagnostic marker of mastocytosis. This includes analysis of blood with highly sensitive techniques when clinical symptoms may indicate underlying mastocytosis. This approach is supported by a reported high sensitivity and specificity,²⁻⁵ and is now included in diagnostic algorithms for suspected mastocytosis.⁶ Presently, mutation analysis of blood is primarily based on laboratory developed tests (LDTs) due to the lack of sufficiently sensitive commercially available *in vitro* diagnostic tests. Commonly used LDTs include allele-specific quantitative PCR (qPCR), reverse transcriptase-PCR (RT-PCR) plus restriction fragment length polymorphism (RFLP) and peptide nucleic acid (PNA)-mediated PCR.⁷ LDTs typically require extensive method validation by every laboratory and specialized laboratory staff. Together, this may limit the availability of blood-based *KIT* D816V mutation analysis. Mastocytosis may therefore go undetected, in particular in patients without skin lesions or elevated tryptase which comprises a significant (and likely underestimated) fraction of patients in larger cohorts.⁸ Missing a diagnosis of mastocytosis may have severe consequences for patients. Currently, there is therefore an unmet need for a commercially available highly sensitive *KIT* D816V mutation analysis to release the potential of blood-based screening in mastocytosis.

We here present the clinical validation of a new highly sensitive qPCR-based *KIT* D816V mutation analysis that was developed in collaboration between Mastocytosis Centre Odense University Hospital (MastOUH) and PentaBase Aps., Odense, Denmark (<https://www.pentabase.com/>). The PentaBase 'PlentiPlex Mastocytosis' assay is now commercially available as CE-IVD labelled *in vitro* diagnostic *KIT* D816V mutation analysis. The study was performed in accordance with the Danish National Committee on Health Research Ethics (with exception from informed consent) and the Helsinki Declaration.

The specificity of the PentaBase-assay was analyzed using DNA from 58 control samples from healthy blood donors. The analysis was performed according to the manufacturer's recommendations (PentaBase-assay technology and protocol described in detail in manufacturer's '*instructions for use*') with the QuantStudio 12K instrument (Applied Biosystems). Briefly, the PentaBase-assay is based on allele-specific qPCR, includes an internal control assay and has a detection limit of 0.01% *KIT* D816V-positive alleles. The PentaBase-assay is available in two versions, with the 'Ready-to-use' being the most simple and the laboratory only needs to add DNA to the tubes with pre-aliquoted master-mix, thereby making the Pentabase-assay simpler than most LDTs, including the MastOUH-assay. Five µl DNA was analyzed per

qPCR well. The total reaction volume was 25 µl. All qPCR runs included NTC control (water), D816V-negative control and D816V-positive control samples.

In this study, none of the 58 control samples fulfilled criteria of positivity. The specificity of the PentaBase-assay was therefore 100% (Figure 1, Supplementary Table S1).

The clinical sensitivity of the PentaBase-assay was analyzed using DNA from 115 clinical follow-up blood samples from known *KIT* D816V mutation-positive MastOUH patients (D816V previously detected in BM, skin, and/or blood). Diagnoses and more detailed inclusion criteria of the patients are listed in Supplementary Table S2. The sensitivity of the PentaBase-assay was further compared to the MastOUH-assay by analyzing DNA from the same extractions using the two assays. The MastOUH-assay is a *KIT* D816V mutation-specific qPCR assay previously described in detail,⁴ including refined criteria for positivity.² These and additional studies describe the validation of the MastOUH-assay,³ including analysis of multiple *KIT* D816V mutation-negative control samples and the MastOUH-assay is therefore validated from a technical perspective. This includes a specificity of 100% and a high clinical sensitivity.^{2-4,9}

In this study, 84 of 115 samples (73%) tested positive with the PentaBase-assay. 89 of 115 samples (77%) tested positive in the first analysis with the MastOUH-assay, thus demonstrating no significant difference in sensitivity between the two assays (Figure 2; chi-square test; $P = 0.45$). The five PentaBase-negative/MastOUH-positive samples had very low allele burdens (mean 0.009%), thus supporting that mutation levels in these samples were below the PentaBase-assay detection limit. Current practice at MastOUH includes re-analysis of samples not fulfilling criteria of positivity, but producing a borderline result, using an optimized protocol with analysis of increased amounts of DNA.² In this study, 14 of 26 samples testing negative using the MastOUH-assay produced the borderline result that leads to re-analysis. When re-analyzed, 11 of the 14 samples tested positive, thus resulting in 100 of 115 samples testing positive (87%) with the MastOUH-assay. When including the result of re-analysis, the MastOUH-assay has a significantly higher sensitivity than the PentaBase-assay (Figure 2; chi-square test; $P = 0.008$). It should be noted, however, that the protocol with re-analysis after additional DNA extraction and DNA upconcentration is specialized and most likely only applied in few laboratories performing *KIT* D816V mutation analysis. For the present purpose, we therefore consider it relevant to compare the PentaBase-assay to the MastOUH-assay standard protocol (without re-analysis) that would be a typical alternative to a commercial test. Here, the PentaBase-assay has the advantage of being a simpler CE-IVD labelled test, although the MastOUH-assay may be optimized to produce a higher sensitivity.

Allele burden data was also compared (Supplementary Figure S1). The two assays produced highly similar results at the absolute scale and a correlation coefficient of 0.98, thus demonstrating highly similar quantitative performance.

The present study thereby demonstrates the new commercial PentaBase-assay to have a specificity of 100% and a clinical meaningful sensitivity (73%) comparable to the MastOUH-assay in the setting where a specialized protocol with re-analysis of samples producing a borderline result is not applied. The PentaBase *KIT* D816V assay is thereby suitable for widespread use in laboratories favoring a commercial CE-IVD test with manufacturer's recommendations for laboratory protocol and interpretation of results and product support. Mastocytosis may pose a diagnostic challenge in the clinic. Detection of the *KIT* D816V mutation in blood has been demonstrated as a strong diagnostic marker in mastocytosis. The PentaBase-assay thereby holds the potential to help release the full value of blood-based *KIT* D816V screening as a tool to facilitate diagnosing and thereby correctly treating mastocytosis patients, although a BM investigation is mandatory in adult patients with highly suspected SM regardless of the *KIT* mutation status.

Acknowledgments

None

Supporting Information

Additional supporting information (Supplementary Table S1, Supplementary Table S2 and Supplementary Figure S1) may be found online in the Supporting Information section at the end of the article.

Disclosure of Conflicts of Interest

TK, Odense University Hospital and the Region of Southern Denmark receive royalty from PentaBase Aps. from the sale of the PentaBase-assay pursuant to the Act on inventions at public research institutions (LBK210 - paragraph 12, section 1 & 2).

Conflicts of interest outside this work: TK received honoraria from Novartis, SBO received honoraria from Thermo Fisher, Blueprint and Novartis, CBJ received research grants from Novartis, Alimmune, HAL Allergy, Allakos, Thermo Fisher, Ga2len and AllergyTherapeutics.

References

1. Horny HP, Metcalfe DD, Akin C, Bennett JM, Arber DA, Bain BJ, Petersen LC, Escribano L, Tefferi A, Valent P. Mastocytosis. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues. Revised 4th ed. Lyon, France: International Agency for Research and Cancer (IARC); 2017: pp 61-69.
2. Kristensen T, Vestergaard H, Bindslev-Jensen C, Møller MB, Broesby-Olsen S. Sensitive KIT D816V mutation analysis of blood as a diagnostic test in mastocytosis. *Am J Hematol*. 2014;89:493-498.
3. Kristensen T, Vestergaard H, Bindslev-Jensen C, Mortz CG, Kjaer HF, Ollert M, Møller MB, Broesby-Olsen S. Prospective evaluation of the diagnostic value of sensitive KIT D816V mutation analysis of blood in adults with suspected systemic mastocytosis. *Allergy* 2017;72:1737-1743.
4. Kristensen T, Vestergaard H, Møller MB. Improved detection of the KIT D816V mutation in patients with systemic mastocytosis using a quantitative and highly sensitive real-time qPCR assay. *J Mol Diagn*. 2011;13:180-188.
5. Jara-Acevedo M, Teodosio C, Sanchez-Muñoz L, Álvarez-Twose I, Mayado A et al. Detection of the KIT D816V mutation in peripheral blood of systemic mastocytosis: diagnostic implications. *Modern Pathology* 2015;28:1138-1149.
6. Valent P, Escribano L, Broesby-Olsen S, Hartmann K, Grattan C, Brockow K, et al. Proposed diagnostic algorithm for patients with suspected mastocytosis: a proposal of the European Competence Network on Mastocytosis. *Allergy* 2014;69:1267-1274.
7. Arock M, Sotlar K, Akin C, Broesby-Olsen S, Hoermann G, et al. KIT mutation analysis in mast cell neoplasms: recommendations of the European Competence Network on Mastocytosis. *Leukemia* 2015;6:1223-1232.
8. Pieri L, Bonadonna P, Elena C, Papayannidis C, Grifoni FI et al. Clinical presentation and management practice of systemic mastocytosis. A survey on 460 Italian patients. *Am J Hematol* 2016;91:692-699.

9. Kristensen T, Broesby-Olsen S, Vestergaard H, Bindslev-Jensen C, Mortz CG, Kjaer HF, Møller MB.

Towards rational diagnostics in mastocytosis: clinical validation of sensitive KIT D816V mutation analysis of unfractionated whole-blood. *Leuk Lymphoma* 2019;60:268-270.

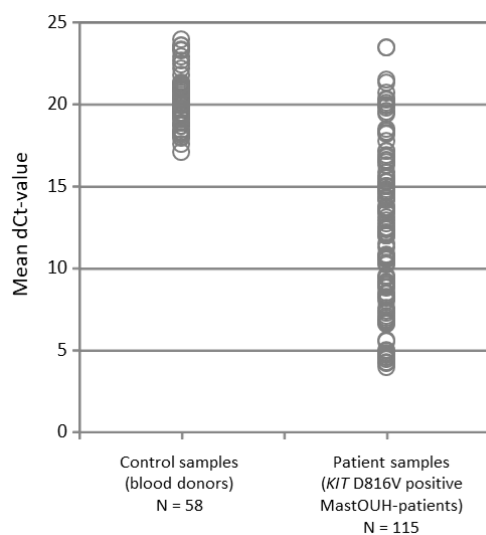


Figure 1. Mean dC_t -values produced by the PentaBase *KIT* D816V assay in 58 control blood samples and 115 patient blood samples. Mean dC_t -values were calculated as the mean of the two lowest C_t -values produced by the mutation-specific assay and subtracted by the C_t -value of the reference assay. A C_t -value of 50 was used for the dC_t -value calculation in reactions producing no C_t -value in 50 cycles. The following criteria of positivity were applied in the PentaBase-assay; 2 or 3 of 3 mutation-specific replicates with a C_t -value < 44.00 and a mean dC_t -value of the two lowest C_t -values produced by the mutation-specific assay < 15.00.

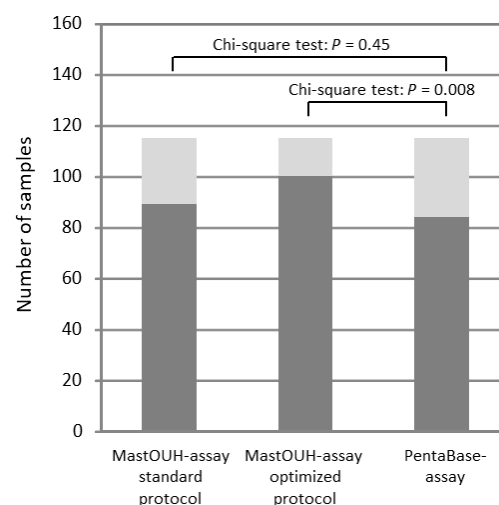
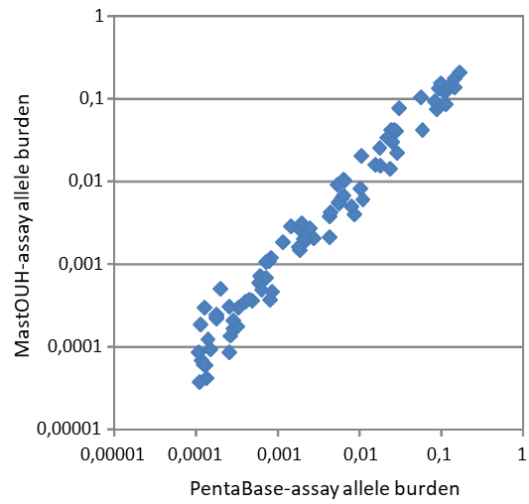


Figure 2. Number of blood samples from *KIT* D816V mutation-positive MastOUH patients fulfilling criteria of positivity when using the MastOUH-assay standard protocol (without re-analysis), the MastOUH-assay optimized protocol (with re-analysis of samples not fulfilling criteria of positivity, but producing a borderline result, using increased amounts of DNA) and the PentaBase assay according to manufacturers' recommendations.



Supplementary Figure S1. The PentaBase-assay *KIT* D816V mutation positive allele burden vs. the MastOUH-assay *KIT* D816V mutation positive allele burden in blood samples testing positive in both assays (N = 84; correlation analysis, $P < 0.001$, $R = 0.98$).

Supplementary Table S1. Ct-values produced by the PentaBase *KIT* D816V assay in 58 control blood samples. None of the samples fulfilled criteria of positivity. The reference assay was analyzed in a single reaction and the *KIT* D816V assay was analyzed in three reactions for each sample. Each reaction included an internal control assay to determine whether amplification has taken place in the well.

Sample ID	Ref Ct	Internal control Ct	D816V Ct #1	Internal control Ct #1	D816V Ct #2	Internal control Ct #2	D816V Ct #3	Internal control Ct #3	Mean dCt*	D816V status
Control 1	27,08	27,17	45,47	27,39	48,64	27,03	Undet.	27,02	19,97	Negative
Control 2	26,12	27,26	48,08	27,53	Undet.	27,92	Undet.	27,66	22,92	Negative
Control 3	26,70	26,94	44,84	27,15	44,52	27,38	49,10	27,16	17,98	Negative
Control 4	27,15	27,48	Undet.	27,44	Undet.	27,12	46,10	27,23	20,90	Negative
Control 5	26,37	26,68	47,67	26,67	47,36	26,77	48,04	26,70	21,14	Negative
Control 6	26,54	26,78	Undet.	27,00	45,01	26,98	Undet.	27,73	20,96	Negative
Control 7	26,43	26,66	48,86	26,77	43,79	27,18	48,03	26,89	19,48	Negative
Control 8	26,47	27,35	46,47	27,22	Undet.	27,71	42,39	27,34	17,96	Negative
Control 9	26,73	26,70	Undet.	26,80	Undet.	26,67	Undet.	26,75	23,27	Negative
Control 10	26,77	27,02	42,14	26,65	47,70	26,42	48,68	26,43	18,15	Negative
Control 11	26,27	27,34	48,74	27,29	Undet.	27,15	45,89	27,36	21,05	Negative
Control 12	26,53	26,89	Undet.	26,83	45,60	26,32	Undet.	26,49	21,27	Negative
Control 13	26,67	26,77	Undet.	27,05	Undet.	27,30	Undet.	27,01	23,33	Negative
Control 14	26,29	26,18	48,01	26,30	45,67	26,31	46,65	26,56	19,87	Negative
Control 15	26,30	27,30	Undet.	27,67	45,23	27,40	49,37	27,35	21,01	Negative
Control 16	26,54	26,47	Undet.	26,48	47,45	26,56	Undet.	26,45	22,19	Negative
Control 17	26,91	26,61	Undet.	26,24	44,93	26,06	45,73	26,25	18,42	Negative
Control 18	25,83	26,88	43,75	27,09	44,40	27,29	Undet.	27,37	18,24	Negative
Control 19	26,98	26,80	47,46	26,72	Undet.	26,63	47,17	26,77	20,33	Negative
Control 20	27,01	26,83	Undet.	27,36	49,36	27,02	Undet.	27,11	22,67	Negative
Control 21	24,62	25,25	42,97	25,65	48,02	25,60	48,14	25,50	20,88	Negative
Control 22	24,21	24,99	48,47	25,35	42,72	25,31	44,34	25,30	19,33	Negative
Control 23	24,24	24,40	43,12	24,57	42,76	24,51	43,18	24,25	18,71	Negative
Control 24	23,92	24,67	44,01	25,23	42,01	25,32	42,38	25,26	18,28	Negative
Control 25	24,04	24,11	45,26	24,32	47,15	24,34	43,29	24,45	20,23	Negative
Control 26	24,28	24,82	43,98	25,02	42,17	25,01	41,55	24,93	17,58	Negative

Control 27	23,90	24,54	44,90	24,97	47,68	24,99	43,65	25,07	20,37	Negative
Control 28	24,58	25,02	42,30	25,55	45,06	25,73	45,50	25,52	19,10	Negative
Control 29	24,35	25,54	44,87	25,77	46,47	26,02	42,76	25,86	19,47	Negative
Control 30	24,31	24,57	Undet.	24,63	44,81	24,16	43,14	24,31	19,66	Negative
Control 31	24,32	24,47	49,28	24,33	48,05	24,43	44,14	24,39	21,77	Negative
Control 32	24,25	24,38	42,10	24,66	45,71	24,97	44,64	24,51	19,12	Negative
Control 33	24,42	25,18	46,09	25,43	Undet.	25,45	45,41	25,59	21,32	Negative
Control 34	24,21	24,94	45,25	25,20	48,29	25,01	48,05	25,17	22,45	Negative
Control 35	24,75	24,98	43,23	25,39	Undet.	25,42	42,72	25,52	18,22	Negative
Control 36	23,78	24,09	42,89	24,40	44,20	24,56	43,48	24,42	19,40	Negative
Control 37	23,99	24,63	44,49	25,25	44,59	25,48	44,90	25,51	20,55	Negative
Control 38	24,31	24,57	45,54	24,35	44,51	24,37	43,47	24,17	19,67	Negative
Control 39	25,38	25,85	Undet.	26,26	47,85	26,12	Undet.	26,11	23,54	Negative
Control 40	25,53	26,69	44,15	27,09	Undet.	27,17	44,58	27,20	18,83	Negative
Control 41	25,49	25,49	49,21	25,76	46,45	25,73	43,12	25,83	19,29	Negative
Control 42	25,43	25,33	46,00	25,50	45,35	25,55	45,65	25,67	20,07	Negative
Control 43	25,48	25,94	45,54	26,33	46,66	26,11	44,70	26,30	19,64	Negative
Control 44	25,91	25,80	49,95	25,72	44,10	25,68	45,10	25,95	18,69	Negative
Control 45	25,59	26,62	49,44	26,69	43,13	26,90	Undet.	26,82	20,69	Negative
Control 46	25,41	25,86	Undet.	26,40	48,01	26,03	Undet.	26,33	23,59	Negative
Control 47	25,77	26,14	47,00	26,28	Undet.	26,49	45,41	26,08	20,44	Negative
Control 48	25,24	25,29	44,43	25,53	44,98	25,51	Undet.	25,69	19,47	Negative
Control 49	25,68	25,49	44,12	25,23	46,67	25,44	Undet.	25,57	19,72	Negative
Control 50	25,09	25,56	49,66	25,75	46,67	25,76	45,12	25,80	20,80	Negative
Control 51	25,70	25,84	46,77	26,05	47,13	26,05	Undet.	26,11	21,25	Negative
Control 52	25,16	25,17	41,98	25,41	42,48	25,40	43,67	25,50	17,07	Negative
Control 53	25,04	25,58	Undet.	25,85	49,14	25,98	48,82	25,77	23,94	Negative
Control 54	25,32	25,29	42,71	25,51	46,80	25,66	48,96	25,92	19,44	Negative
Control 55	25,66	26,02	46,01	26,74	47,24	26,64	44,26	26,64	19,48	Negative
Control 56	26,11	25,97	45,57	25,95	Undet.	26,29	49,11	26,05	21,23	Negative
Control 57	25,58	25,74	48,92	26,26	44,15	26,26	Undet.	26,23	20,96	Negative
Control 58	25,42	26,61	42,67	27,32	44,68	27,13	44,84	27,35	18,25	Negative

A threshold of 0.3 was applied in all qPCR runs for the reference and D816V assays (approx. 10% of the signal strength at cycle 45 for the reference assay).
A threshold of 0.03 was applied in all qPCR runs for the internal control assay.

Abbreviations: Ref = Reference, Undet. = Undetermined (qPCR reactions in which no Ct-value is produced in 50 cycles of PCR).

*Mean dCt-values were calculated as the mean of the two lowest Ct-values produced by the *KIT* D816V mutation-specific assay and subtracted by the Ct-value of the reference assay. A Ct-value of 50 was used for the dCt-value calculation in reactions producing no Ct-value in 50 cycles. The following criteria of positivity were applied in the PentaBase-assay; 2 or 3 of 3 mutation-specific replicates with a Ct-value < 44.00 and a mean dCt-value of the two lowest Ct-values produced by the mutation-specific assay < 15.00.

Supplementary Table S2. Ct-values produced by the PentaBase-assay and the MastOUH-assay in 115 patient blood samples (ISM skin+: 73, BMM: 24, SM-AHN: 7, SSM: 5, MIS: 3, MMCAS: 2, CM: 1). All 115 patients were known *KIT* D816V mutation positive based a previous mutation-positive analysis of BM, skin and/or blood. The reference assay was analyzed in a single reaction and the *KIT* D816V assay was analyzed in three reactions for each sample. Each reaction included an internal control assay to determine whether amplification has taken place in the well.

Sample ID	Diagnosis	PentaBase-assay											MastOUH-assay	
		Ref Ct	Internal control	D816V Ct #1	Internal control	D816V Ct #2	Internal control	D816V Ct #3	Internal control	Mean dCt*	D816V status	Allele-burden	D816V status	Allele-burden
Patient 1	ISM skin+	25,46	26,25	29,63	26,69	29,63	26,63	29,66	26,73	4,17	Positive	0,14896	Positive	0,17101
Patient 2	SSM	25,95	26,13	30,70	26,24	30,33	25,99	30,77	26,19	4,56	Positive	0,11618	Positive	0,08437
Patient 3	ISM skin+	25,83	25,50	32,54	25,36	32,97	25,54	32,42	25,25	6,64	Positive	0,02910	Positive	0,02200
Patient 4	ISM skin+	26,24	25,89	39,98	25,90	42,11	25,89	39,94	25,81	13,71	Positive	0,00027	Positive	0,00013
Patient 5	ISM skin+	26,14	27,00	Undet.	27,20	44,86	27,19	Undet.	27,13	21,29	Negative		Negative	
Patient 6	ISM skin+	26,39	26,45	37,01	26,26	36,97	26,45	37,13	26,44	10,61	Positive	0,00214	Positive	0,00200
Patient 7	ISM skin+	26,48	26,06	43,32	25,91	40,92	25,80	41,98	25,83	14,97	Positive	0,00012	Positive	0,00007
Patient 8	ISM skin+	26,19	26,25	36,35	26,17	37,23	26,10	36,37	26,00	10,17	Positive	0,00283	Positive	0,00202
Patient 9	ISM skin+	26,30	27,15	35,38	27,64	35,51	27,53	35,58	27,63	9,15	Positive	0,00559	Positive	0,00551
Patient 10	ISM skin+	26,17	26,35	32,91	26,58	32,83	26,70	33,23	26,77	6,70	Positive	0,02834	Positive	0,04024
Patient 11	BMM	26,29	26,96	43,33	27,28	49,23	27,24	48,84	27,18	19,80	Negative		Positive	0,00008
Patient 12	ISM skin+	27,40	27,71	Undet.	27,43	43,78	27,33	42,19	27,19	15,58	Negative		Positive	0,00012
Patient 13	MMCAS	26,80	27,11	45,58	27,44	42,33	27,63	Undet.	27,52	17,16	Negative		Negative	
Patient 14	SSM	25,07	24,90	30,03	24,63	30,00	24,60	29,99	24,58	4,92	Positive	0,08926	Positive	0,07276
Patient 15	ISM skin+	27,28	27,11	36,30	27,05	36,37	27,08	36,23	27,05	8,99	Positive	0,00643	Positive	0,01027
Patient 16	ISM skin+	27,52	27,59	40,52	27,50	41,39	27,66	43,62	27,63	13,43	Positive	0,00034	Positive	0,00030
Patient 17	ISM skin+	27,17	27,16	33,91	27,45	33,72	27,42	33,77	27,31	6,58	Positive	0,03155	Positive	0,07495
Patient 18	BMM	26,11	26,56	42,15	26,80	40,62	27,01	47,25	26,75	15,28	Negative		Negative**	
Patient 19	BMM	29,67	29,61	Undet.	29,59	Undet.	29,59	Undet.	29,57	20,33	Negative		Negative**	
Patient 20	ISM skin+	28,50	28,69	Undet.	28,64	Undet.	28,98	Undet.	28,64	21,50	Negative		Negative**	
Patient 21	BMM	26,97	27,01	44,65	27,23	45,70	27,32	46,24	27,14	18,21	Negative		Negative**	
Patient 22	BMM	26,55	26,28	Undet.	26,46	Undet.	26,46	Undet.	26,44	23,45	Negative		Negative	
Patient 23	MMCAS	24,49	24,96	49,24	24,72	43,02	24,40	42,42	24,69	18,23	Negative		Negative	
Patient 24	ISM skin+	26,17	26,47	33,44	26,48	33,73	26,73	33,66	26,82	7,38	Positive	0,01803	Positive	0,02529

Patient 25	BMM	26,53	26,58	Undet.	26,58	Undet.	26,62	Undet.	26,55	23,47	Negative		Negative	
Patient 26	ISM skin+	27,90	27,98	38,37	27,69	39,25	27,83	38,85	27,63	10,71	Positive	0,00209	Positive	0,00178
Patient 27	BMM	25,72	25,37	39,53	25,72	40,51	25,79	41,10	25,98	14,30	Positive	0,00018	Positive	0,00022
Patient 28	ISM skin+	26,89	26,68	46,70	26,60	41,33	26,60	Undet.	26,55	17,13	Negative		Negative	
Patient 29	BMM	28,35	28,24	Undet.	27,88	45,54	28,06	Undet.	27,84	19,42	Negative		Negative	
Patient 30	ASM-AHN (CMML)	27,99	27,56	40,11	27,77	39,42	27,84	39,01	27,77	11,23	Positive	0,00148	Positive	0,00284
Patient 31	ISM skin+	27,23	27,36	44,33	27,08	Undet.	27,10	Undet.	27,05	19,93	Negative		Negative**	
Patient 32	ISM skin+	26,31	26,15	38,63	25,98	38,96	26,42	39,00	26,13	12,48	Positive	0,00061	Positive	0,00071
Patient 33	ISM skin+	26,16	26,38	38,59	26,40	38,73	26,75	38,77	26,58	12,50	Positive	0,00060	Positive	0,00059
Patient 34	BMM	26,93	27,04	43,89	26,79	Undet.	26,57	43,74	26,66	16,89	Negative		Negative**	
Patient 35	ISM skin+	26,35	26,59	41,53	26,81	41,14	26,77	41,79	27,04	14,99	Positive	0,00012	Positive	0,00018
Patient 36	ASM-AHN (CMML)	25,70	25,85	36,37	25,97	36,53	25,93	36,26	25,99	10,62	Positive	0,00208	Positive	0,00257
Patient 37	BMM	26,50	26,60	49,07	26,37	Undet.	26,11	40,58	26,30	18,33	Negative		Negative	
Patient 38	BMM-AHN (CMML)	26,95	27,62	34,05	27,73	34,34	27,91	33,73	27,48	6,94	Positive	0,02473	Positive	0,04173
Patient 39	CM	27,16	27,98	43,45	27,91	Undet.	28,17	41,88	27,91	15,51	Negative		Negative	
Patient 40	BMM	25,95	25,74	43,94	26,01	40,01	25,95	41,00	26,06	14,55	Positive	0,00015	Positive	0,00009
Patient 41	ISM skin+	26,61	26,37	37,48	26,38	37,32	26,60	37,76	26,55	10,80	Positive	0,00189	Positive	0,00263
Patient 42	SSM	25,76	25,61	31,81	26,15	31,25	25,79	31,46	25,90	5,60	Positive	0,05801	Positive	0,10321
Patient 43	BMM	25,85	26,73	41,00	27,10	41,12	26,95	41,48	27,04	15,21	Negative		Positive	0,00013
Patient 44	ISM skin+	26,42	26,68	33,62	26,59	33,47	26,69	33,51	26,71	7,06	Positive	0,02243	Positive	0,03360
Patient 45	ISM skin+	25,88	26,05	31,03	26,20	30,88	26,08	30,88	26,13	5,01	Positive	0,08635	Positive	0,09224
Patient 46	ISM skin+	27,08	27,19	34,80	26,75	34,77	26,59	34,56	26,65	7,59	Positive	0,01612	Positive	0,01579
Patient 47	ISM skin+	25,67	26,15	38,81	26,36	40,12	26,28	40,00	26,30	13,73	Positive	0,00026	Positive	0,00030
Patient 48	ISM skin+	25,96	26,07	37,97	26,06	38,38	26,13	37,91	26,08	11,97	Positive	0,00085	Positive	0,00119
Patient 49	ISM skin+	26,36	27,11	31,17	27,33	31,22	27,41	30,94	27,19	4,69	Positive	0,10786	Positive	0,13559
Patient 50	ASM-AHN (MM)	26,29	26,06	30,30	25,61	30,70	26,03	30,26	25,71	3,99	Positive	0,17198	Positive	0,20189
Patient 51	ISM skin+	26,67	26,82	30,94	26,30	31,03	26,39	30,86	26,35	4,23	Positive	0,14808	Positive	0,13644
Patient 52	ISM skin+	26,33	26,50	38,01	26,63	38,50	26,41	37,61	26,57	11,48	Positive	0,00119	Positive	0,00182
Patient 53	ISM skin+	26,99	26,59	35,56	26,66	35,17	26,65	35,23	26,82	8,22	Positive	0,01063	Positive	0,02026
Patient 54	ISM skin+	25,15	25,54	34,19	25,43	34,47	25,74	34,36	25,64	9,13	Positive	0,00550	Positive	0,00906
Patient 55	ISM skin+	26,65	26,78	40,55	26,31	39,97	26,16	41,20	25,99	13,61	Positive	0,00029	Positive	0,00016
Patient 56	ISM skin+	26,03	25,90	36,30	25,73	37,59	25,96	37,09	25,66	10,66	Positive	0,00204	Positive	0,00309
Patient 57	BMM	26,52	26,13	40,15	25,76	41,95	25,64	39,10	25,66	13,10	Positive	0,00041	Positive	0,00035

Patient 58	ISM skin+	25,87	26,19	32,68	25,99	32,57	25,96	32,71	26,03	6,76	Positive	0,02695	Positive	0,04080
Patient 59	SSM	25,71	25,92	30,65	25,84	30,31	25,78	30,30	25,68	4,59	Positive	0,11344	Positive	0,11914
Patient 60	BMM-AHN (AML)	26,46	27,21	43,06	26,97	Undet.	27,02	Undet.	27,01	20,08	Negative		Negative	
Patient 61	BMM	25,96	25,80	46,25	25,65	45,19	25,48	45,83	25,52	19,55	Negative		Positive	0,00010
Patient 62	BMM	25,73	25,86	44,19	25,88	47,80	25,86	44,21	25,75	18,46	Negative		Negative	
Patient 63	ISM skin+	25,82	25,68	40,17	25,54	40,60	25,49	39,72	25,66	14,13	Positive	0,00020	Positive	0,00050
Patient 64	BMM	26,17	26,16	38,53	26,03	39,80	25,92	37,72	25,88	11,96	Positive	0,00087	Positive	0,00046
Patient 65	ISM skin+	26,07	25,89	37,16	25,84	36,98	25,83	36,69	25,85	10,77	Positive	0,00190	Positive	0,00144
Patient 66	SM-AHN (MDS)***	24,66	24,53	48,67	24,78	47,15	24,62	43,55	24,74	20,69	Negative		Negative	
Patient 67	BMM	25,13	24,94	40,98	25,05	43,03	25,08	41,81	25,15	16,27	Negative		Negative**	
Patient 68	ISM skin+	25,29	24,97	39,50	25,32	39,62	25,02	39,61	25,12	14,26	Positive	0,00018	Positive	0,00024
Patient 69	ISM skin+	24,67	25,42	34,14	25,91	34,07	25,94	34,33	25,96	9,43	Positive	0,00442	Positive	0,00208
Patient 70	ISM skin+	25,16	25,04	35,71	25,06	35,70	25,16	35,58	25,14	10,48	Positive	0,00223	Positive	0,00205
Patient 71	MIS	25,25	25,03	42,33	25,16	41,75	25,01	42,87	25,12	16,79	Negative		Positive	0,00002
Patient 72	ASM-AHN (CMML)	25,17	25,50	37,53	25,69	36,88	25,76	37,67	25,70	12,04	Positive	0,00080	Positive	0,00108
Patient 73	BMM	25,51	25,79	Undet.	26,25	40,77	25,99	Undet.	26,21	19,87	Negative		Negative**	
Patient 74	BMM	25,92	25,51	35,58	25,71	35,73	25,78	35,29	25,65	9,51	Positive	0,00436	Positive	0,00375
Patient 75	ISM skin+	25,78	26,00	38,03	26,01	38,28	26,17	38,64	26,00	12,37	Positive	0,00065	Positive	0,00049
Patient 76	SSM	24,54	24,33	31,90	24,75	31,79	24,54	31,83	24,70	7,27	Positive	0,01847	Positive	0,01516
Patient 77	ISM skin+	25,29	25,04	44,14	25,30	40,74	25,44	41,60	25,30	15,88	Negative		Negative**	
Patient 78	ISM skin+	25,16	25,97	33,35	26,27	33,30	26,19	33,53	26,30	8,16	Positive	0,01042	Positive	0,00802
Patient 79	ISM skin+	24,59	24,56	40,80	24,95	38,90	24,84	39,64	25,10	14,68	Positive	0,00014	Positive	0,00006
Patient 80	ISM skin+	24,56	24,41	32,91	24,42	33,07	24,56	33,00	24,54	8,40	Positive	0,00875	Positive	0,00398
Patient 81	BMM	24,38	25,15	39,45	25,79	39,27	25,96	38,79	25,97	14,65	Positive	0,00014	Positive	0,00004
Patient 82	ISM skin+	24,91	24,51	38,47	24,46	38,72	24,47	38,76	24,44	13,69	Positive	0,00026	Positive	0,00008
Patient 83	ISM skin+	24,25	24,43	31,25	24,64	30,97	24,39	31,32	24,76	6,86	Positive	0,02412	Positive	0,01401
Patient 84	ISM skin+	25,41	26,05	39,97	26,54	39,37	26,66	40,45	26,55	14,25	Positive	0,00018	Positive	0,00023
Patient 85	ISM skin+	25,31	25,07	34,36	25,20	34,25	25,08	34,34	25,28	8,98	Positive	0,00609	Positive	0,00618
Patient 86	MIS	24,98	25,86	36,97	25,97	36,97	26,04	37,17	26,13	11,99	Positive	0,00082	Positive	0,00037
Patient 87	ISM skin+	25,34	25,43	34,42	25,72	34,40	25,70	34,42	25,76	9,07	Positive	0,00573	Positive	0,00534
Patient 88	ISM skin+	25,61	26,37	40,29	26,70	42,67	26,81	40,20	26,84	14,64	Positive	0,00014	Positive	0,00012
Patient 89	ISM skin+	23,79	23,79	37,07	23,98	37,25	24,11	37,14	24,26	13,32	Positive	0,00033	Positive	0,00017
Patient 90	ISM skin+	25,75	26,01	44,46	25,87	43,44	25,95	41,27	25,90	16,60	Negative		Negative**	

Patient 91	ISM skin+	24,97	24,95	41,76	25,30	39,66	24,95	40,26	25,39	14,99	Positive	0,00011	Positive	0,00009
Patient 92	ISM skin+	25,94	25,69	36,06	25,33	36,66	25,53	36,48	25,79	10,33	Positive	0,00253	Positive	0,00264
Patient 93	ISM skin+	25,51	26,46	37,54	26,58	37,83	26,52	37,99	26,58	12,17	Positive	0,00073	Positive	0,00068
Patient 94	ISM skin+	24,51	24,98	32,77	25,29	32,32	24,91	32,74	25,24	8,02	Positive	0,01126	Positive	0,00600
Patient 95	ISM skin+	25,06	26,20	41,89	26,44	41,39	26,22	42,81	26,43	16,58	Negative		Negative**	
Patient 96	BMM	24,80	24,94	37,40	25,23	37,70	25,33	37,62	25,00	12,72	Positive	0,00050	Positive	0,00036
Patient 97	BMM	24,94	25,79	39,13	26,11	40,97	26,19	40,68	26,38	14,97	Positive	0,00011	Positive	0,00004
Patient 98	ISM skin+	24,72	24,62	41,29	24,93	46,96	24,89	41,09	24,87	16,47	Negative		Negative**	
Patient 99	ISM skin+	24,98	25,75	30,59	26,28	30,48	26,25	30,50	26,36	5,51	Positive	0,06018	Positive	0,04116
Patient 100	ISM skin+	24,41	24,66	34,95	25,18	35,29	25,13	35,35	25,28	10,71	Positive	0,00188	Positive	0,00157
Patient 101	ISM skin+	24,64	25,62	33,21	26,12	33,07	26,05	33,30	26,24	8,50	Positive	0,00822	Positive	0,00497
Patient 102	ISM skin+	24,59	24,98	39,03	25,40	37,90	25,47	38,27	25,51	13,50	Positive	0,00030	Positive	0,00021
Patient 103	BMM	25,50	25,28	37,70	25,69	38,42	25,80	37,63	25,68	12,17	Positive	0,00074	Positive	0,00105
Patient 104	ISM skin+	25,04	25,45	32,09	26,03	31,80	25,71	31,91	25,87	6,81	Positive	0,02547	Positive	0,02986
Patient 105	ISM skin+	25,59	25,80	45,89	25,74	40,75	25,80	45,94	25,95	17,73	Negative		Negative**	
Patient 106	BMM	25,28	25,49	38,51	25,56	38,10	25,41	38,25	25,81	12,90	Positive	0,00045	Positive	0,00037
Patient 107	ISM skin+	24,85	24,76	33,98	25,13	33,63	24,98	33,69	24,90	8,82	Positive	0,00669	Positive	0,01007
Patient 108	ISM skin+	24,87	25,29	40,72	25,34	39,94	25,34	39,40	25,45	14,80	Positive	0,00013	Positive	0,00006
Patient 109	ISM skin+	24,83	25,16	29,70	25,19	29,59	25,25	29,88	25,47	4,81	Positive	0,09535	Positive	0,13092
Patient 110	MIS	24,58	24,56	39,96	24,86	38,64	24,96	40,40	24,95	14,72	Positive	0,00013	Positive	0,00029
Patient 111	ISM skin+	25,21	25,04	34,55	25,11	34,73	25,01	34,92	25,24	9,43	Positive	0,00450	Positive	0,00413
Patient 112	ISM skin+	25,33	25,19	49,78	25,47	42,48	25,57	48,67	25,79	20,25	Negative		Negative**	
Patient 113	ISM skin+	25,02	26,22	33,93	26,78	33,88	26,62	34,09	26,95	8,88	Positive	0,00643	Positive	0,00673
Patient 114	ISM skin+	24,84	24,75	29,61	25,30	29,16	25,10	29,36	25,23	4,42	Positive	0,12390	Positive	0,13408
Patient 115	ISM skin+	25,28	25,66	30,03	26,03	30,05	25,99	30,18	26,09	4,77	Positive	0,09964	Positive	0,15300

A threshold of 0.3 was applied in all qPCR runs for the reference and D816V assays (approx. 10% of the signal strength at cycle 45 for the reference assay).

A threshold of 0.03 was applied in all qPCR runs for the internal control assay.

Allele burden represents the fraction of *KIT* gene alleles carrying the D816V mutation. Allele burden in the PentaBase-assay is calculated using a PCR efficiency of 100% and 94% for the reference and D816V assays, respectively.

The patients included in the present study were also included in a previous MastOUH-study (Kristensen et al., Leuk Lymphoma 2019;60:268-270). The previous study included a total of 164 samples from known KIT D816V mutation-positive MastOUH patients (D816V previously detected in BM, skin, and/or blood). However, only 65 of the 164 samples from the previous study had residual DNA available for inclusion in the present study. These 65 samples were all included in the present study. No residual DNA was available for the remaining 99 of the 164 samples from the previous study. For these 99 patient samples, it was instead possible to include DNA from a follow-up blood sample in a total of 50 patient samples thus reaching the total of 115 patient samples in the present study. All samples consisted of DNA from NH4Cl-hemolysed total leucocyte preparations.

For BMM (N = 24) and ISM skin+ (N = 73), subgroup analysis showed no significant difference for the PentaBase-assay vs. the MastOUH-assay standard protocol (without re-analysis of borderline samples). However, when comparing the PentaBase-assay vs. the optimized MastOUH-protocol (with re-analysis of borderline samples), the MastOUH-assay has a significantly higher sensitivity than the PentaBase-assay in the BMM group (chi-square test; P = 0.02) and a non-significant tendency to a higher sensitivity of the MastOUH-assay was observed in the ISM skin+ group (chi-square test; P = 0.06), thus reflecting the results of the overall group of 115 patients as described in the main manuscript. Due to low sample sizes (N = 1 – 7), no statistical analysis was performed in the remaining subgroups (SM-AHN, SSM, MIS, MMCAS and CM).

Abbreviations: AHN = associated hematological neoplasm, AML = acute myeloid leukemia, ASM = aggressive SM, BMM = bone marrow mastocytosis, CM = cutaneous mastocytosis, CMML = chronic myelomonocytic leukemia, ISM skin+ = indolent systemic mastocytosis (SM) with skin lesion, MDS = myelodysplastic syndrome, MIS = mastocytosis in the skin (bone marrow biopsy not performed), MM = multiple myeloma, MMCAS = KIT D816V-positive monoclonal mast cell activation syndrome, Ref = Reference, SSM = smouldering SM, Undet. = Undetermined (qPCR reactions in which no Ct-value is produced in 50 cycles of PCR).

*Mean dCt-values were calculated as the mean of the two lowest Ct-values produced by the *KIT* D816V mutation-specific assay and subtracted by the Ct-value of the reference assay. A Ct-value of 50 was used for the dCt-value calculation in reactions producing no Ct-value in 50 cycles. The following criteria of positivity were applied in the PentaBase-assay; 2 or 3 of 3 mutation-specific replicates with a Ct-value < 44.00 and a mean dCt-value of the two lowest Ct-values produced by the mutation-specific assay < 15.00.

A dCt-value of 15.00 corresponds to an allele burden of 0.01%, which is therefore the calculated detection limit of the PentaBase-assay.

**Samples not fulfilling criteria of positivity, but producing a borderline result that leads to re-analysis using increased amounts of DNA in line with current clinical practice at MastOUH (as previously described in Kristensen et al. 2014, Am J Hematol 89:493-98). Of the 14 samples that produced the borderline result, 11 samples fulfilled criteria of positivity in re-analysis (patient no. 18, 19, 20, 21, 67, 73, 77, 90, 95, 98, 112).

***SM not classified in patient no. 66.