Systemic mastocytosis:

Flow cytometry and Molecular Biology

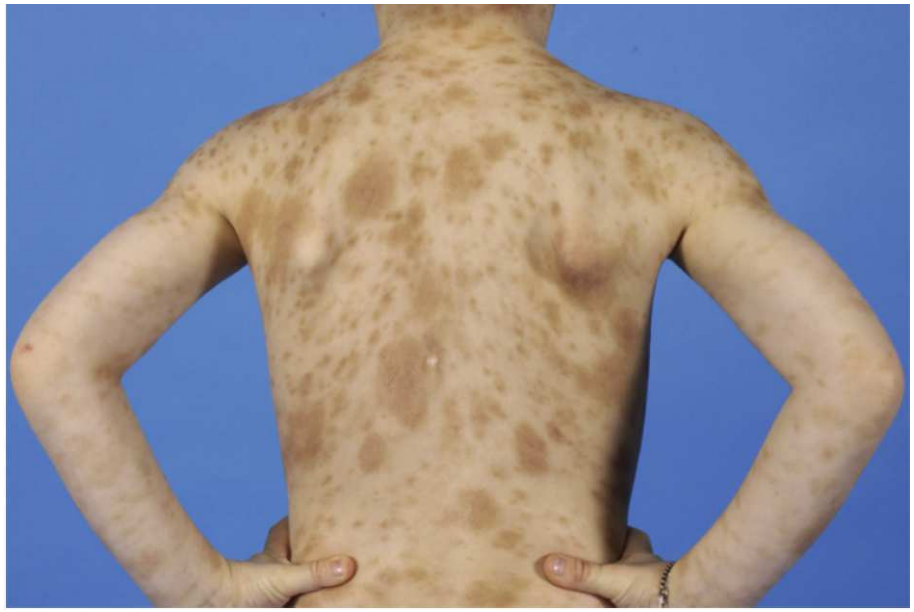
Katrien Vermeulen 21 oktober 2016

UZA

Cuteanous mastocytosis

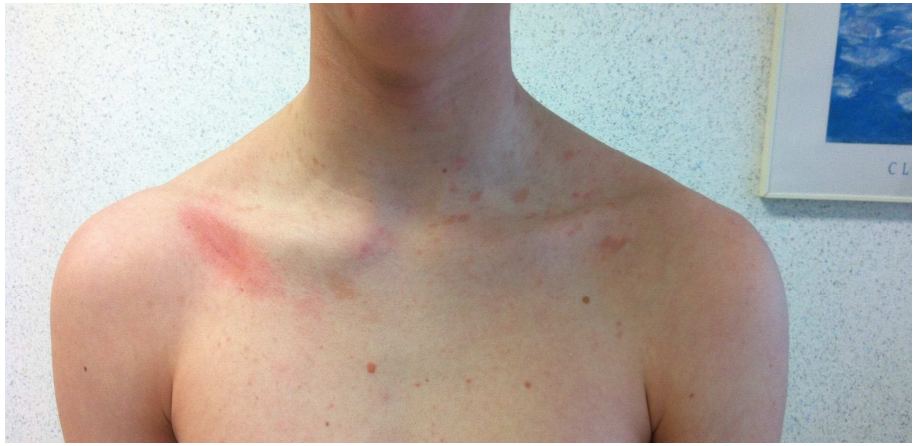
Major criterion

Typical skin lesions of mastocytosis associated with Darier's sign



Minor criteria

- Increased numbers of mast cells in biopsy sections of lesional skin
- KIT mutation in lesional skin tissue
- Absence of criteria for systemic mastocytosis



Systemic mastocytosis

- Rare disease: listed by the office of rare diseases of the National institutes of Health (NIH)
- Heterogenous disease
- Accumulation of neoplastic mast cells in bone marrow and/or organs/tissues
  - Abnormal morpholgy
  - Abnormal immuunphenotype
  - Mutations in KIT D816V in 80% of all systemic mastocytosis

Systemic mastocytosis: hallmarks

- Most patients: indolent disease variant
  - Near normal life expectancy

- Some patients: advanced disease
  - Reduced life expectancy
    - Aggressive systemic mastocytosis
    - Associated hematologic disease (p.e leukemia)
    - Mast cell leukemia
  - Cytoreductive therapy
  - Only curative therapy: allogeneic stem cell transplantation
    - Few new drugs show beneficial effects in advanced disease

WHO classification: Myeloproliferative neoplasms (MPN)

WHO 2008	WHO update 2016
1.Chronic myelogenous leukemia, BCR-ABL1$^+$	1.Chronic myeloid leukemia, BCR-ABL1$^+$
2.Chronic neutrophilic leukaemia	2.Chronic neutrophilic leukaemia
3.Polycythaemia vera	3.Polycythaemia vera
4.Primary myelofibrosis	4.Primary myelofibrosis
5.Essential thrombocytaemia	5.Essential thrombocytaemia
6.Chronic eosinophilic leukaemia, NOS	6.Chronic eosinophilic leukaemia, NOS
7.Mastocytosis	
8.Myeloproliferative neoplasms, unclassifiable	7.Myeloproliferative neoplasms, unclassifiable

Arber et al Blood 2016

Myeloid neoplasms WHO update 2016

WHO 2008	WHO update 2016
1. Myeloproliferative neoplasms	1.Myeloproliferative neoplasms (MPN)
	2.Mastocyosis
2. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB or FGFR1, or with PCM1-JAK2	3.Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB or FGFR1, or with PCM1-JAK2
3. Myelodysplastic/myeloproliferative neoplasms (MDS/MPN)	4.Myelodysplastic/myeloproliferative neoplasms (MDS/MPN)
4. Myelodysplastic syndromes (MDS)	5.Myelodysplastic syndromes (MDS)
5. Acute myeloid leukaemia (AML) and related precursor neoplasms	6.Acute myeloid leukaemia (AML) and related precursor neoplasms

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Mastocytosis: WHO classification

WHO 2008	WHO update 2016
1. Cutaneous mastocytosis (CM)	**1. Cutaneous mastocytosis (CM)**
2. Systemic mastocytosis (SM)	**2. Systemic mastocytosis (SM)**
2.1. Indolent systemic mastocytosis (ISM)	2.1. Indolent systemic mastocytosis (ISM)
2.1.1 Bone marrow mastocytosis 2.1.2 Smouldering mastocytosis	2.2. Smoldering systemic mastocytosis (SSM)
2.2. Systemic mastocytosis with an associated clonal hematological non-mast cell lineage disease (SM-AHNMD)	2.3. Systemic mastocytosis with an associated hematological neoplasm (SM-AHN)
2.3. Aggressive systemic mastocytosis (ASM)	2.4. Aggressive systemic mastocytosis (ASM)
2.4. Mast cell leukemia (MCL)	2.5. Mast cell leukemia (MCL)
2.5. Mast cell sarcoma (MCS)	**3. Mast cell sarcoma (MCS)**
2.6. Extracutaneous mastocytoma	

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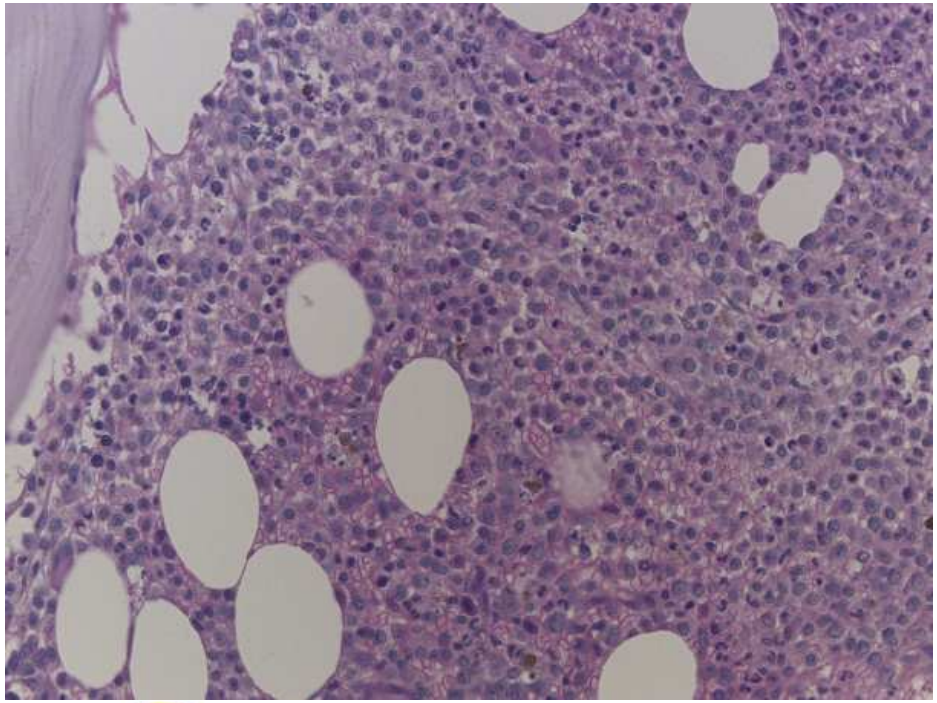
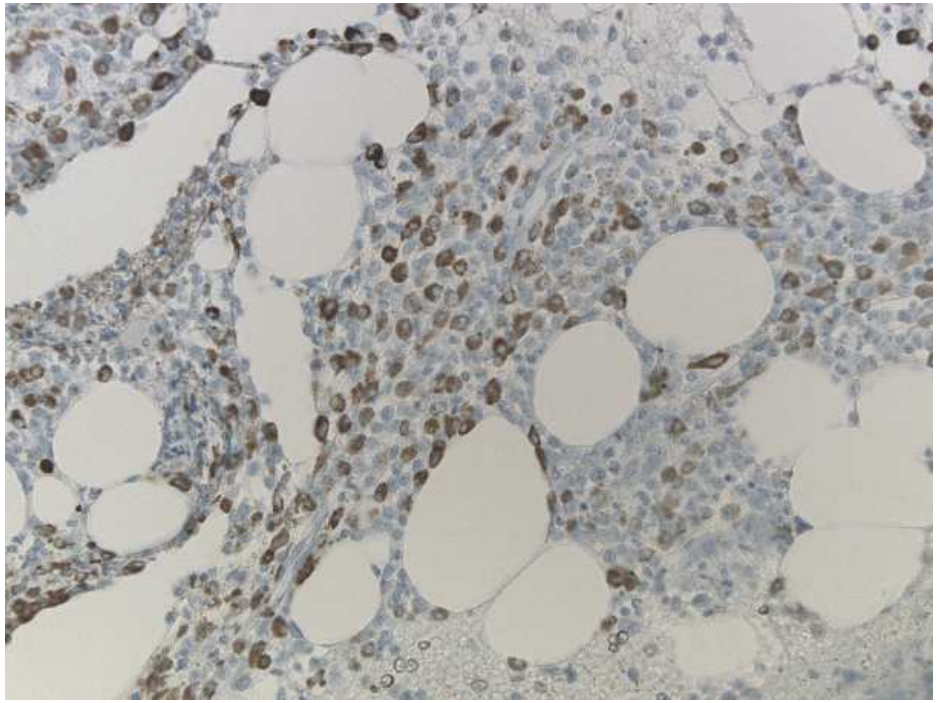
WHO Major criterion: histology

1 major and 1 minor criterion OR 3 minor criteria for diagnosis

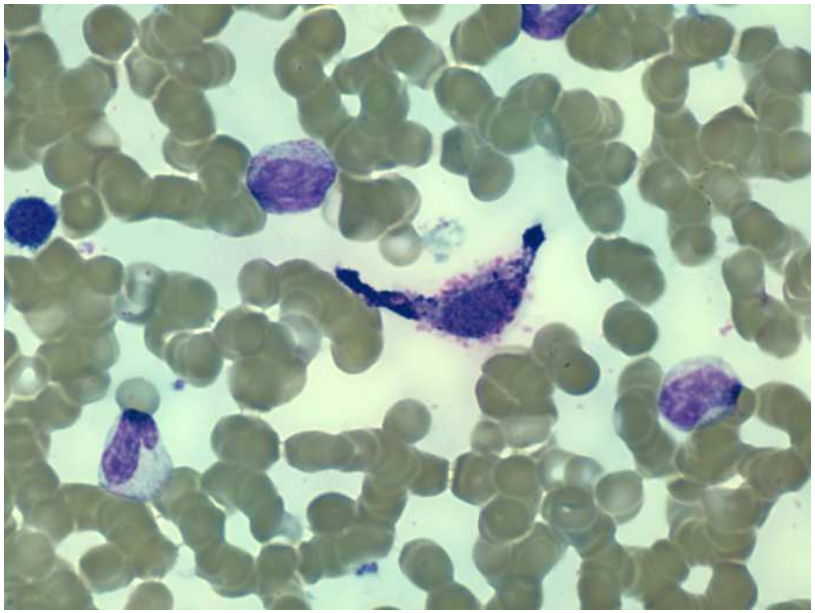
Major criterion: *"Multifocale, dense infiltrates of mast cells (≥ 15% mast cells in aggregates) detected in sections of bone marrow and/or other extracutaneous organ(s)"*

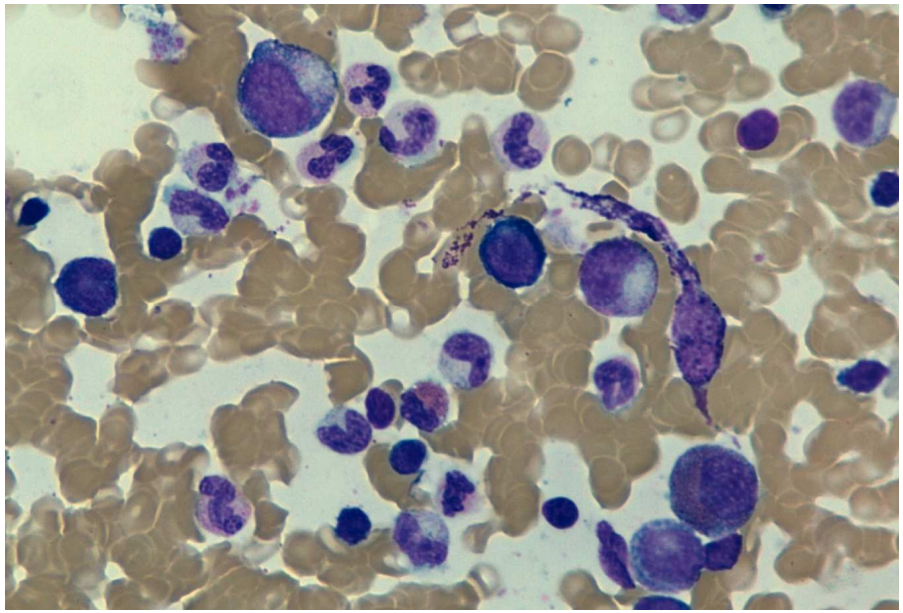




WHO Minor criteria

1. In biopsy sections of bone marrow, >25% of the mast cells are spindle-shaped or have atypical morphology





WHO Minor criteria

2. Detection of an activating point mutation at codon 816 of KIT

- *PCR*

3. Mast cells express CD2 and/or CD25

- *Flow cytometry*

4. Serum tryptase persistenly exceeds 20 ng/ml

- *Fluoro Enzymo Immuno Assay (FEIA) (ImmunoCAP)*

1 major and 1 minor criterion OR 3 minor criteria for diagnosis

MULTIDISCIPLINARY APPROACH!

Standard and guidelines

European Journal of Clinical Investigation (2007) **37**, 435–453

Review

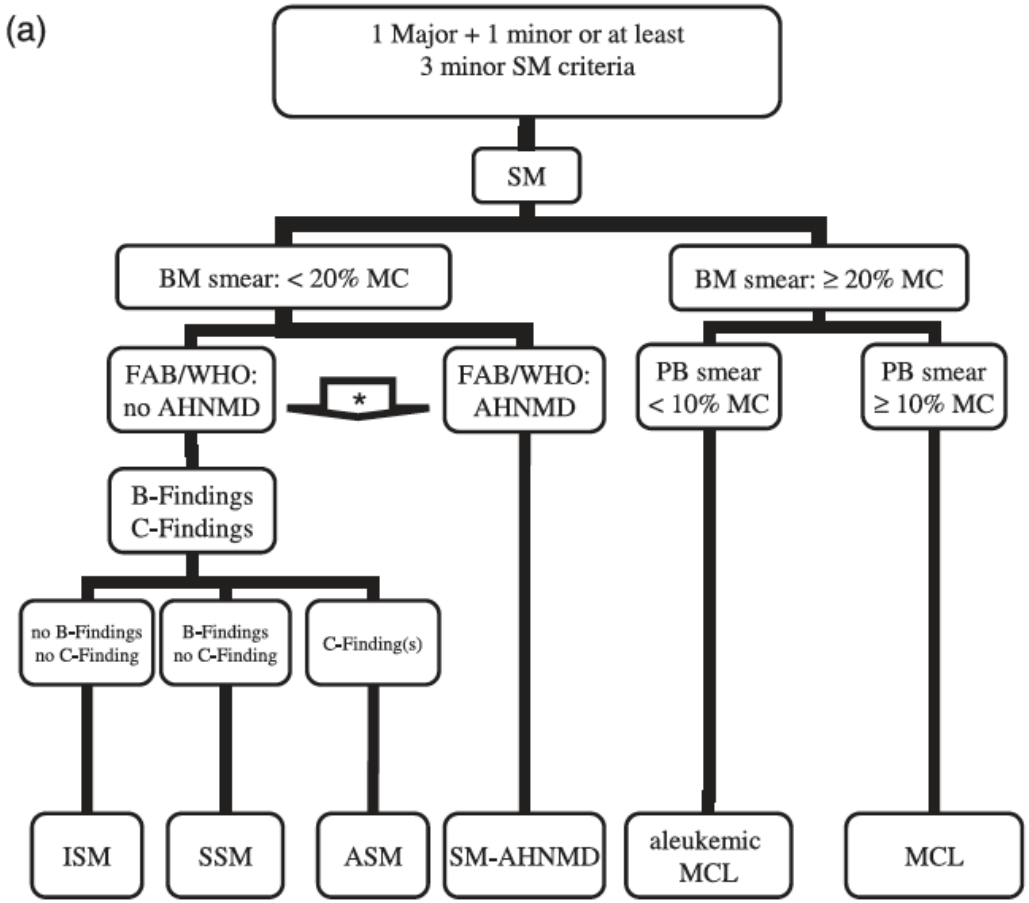
Standards and standardization in mastocytosis: Consensus Statements on Diagnostics, Treatment Recommendations and Response Criteria

P. Valent, C. Akin, L. Escribano, M. Födinger, K. Hartmann, K. Brockow, M. Castells, W. R. Sperr, H. C. Kluin-Nelemans, N. A. T. Hamdy, O. Lortholary, J. Robyn, J. van Doormaal, K. Sotlar, A. W. Hauswirth, M. Arock, O. Hermine, A. Hellmann, M. Triggiani, M. Niedoszytko, L. B. Schwartz, A. Orfao, H.-P. Horny, D. D. Metcalfe

Proposed diagnostic algorithm for patients with suspected mastocytosis: a proposal of the European Competence Network on Mastocytosis

P. Valent[1], L. Escribano[2], S. Broesby-Olsen[3], K. Hartmann[4], C. Grattan[5], K. Brockow[6], M. Niedoszytko[7], B. Nedoszytko[8], J. N. G. Oude Elberink[9], T. Kristensen[10], J. H. Butterfield[11], M. Triggiani[12], I. Alvarez-Twose[13], A. Reiter[14], W. R. Sperr[1], K. Sotlar[15], S. Yavuz[16], H. C. Kluin-Nelemans[17], O. Hermine[18], D. Radia[19], J. J. van Doormaal[9], J. Gotlib[20], A. Orfao[2], F. Siebenhaar[21], L. B. Schwartz[22], M. Castells[23], M. Maurer[21], H.-P. Horny[15], C. Akin[23], D. D. Metcalfe[24] & M. Arock[25]

Algorithm sub-variants of systemic mastocytosis



The immunophenotype of mast cells and its utility in the diagnostic work-up of systemic mastocytosis

Cristina Teodosio, Andrea Mayado,* Laura Sánchez-Muñoz,† José M. Morgado,† María Jara-Acevedo,* Ivan Álvarez-Twose,† Andrés C. García-Montero,* Almudena Matito,† Caldas Caldas,* Luis Escribano,*,1 and Alberto Orfao*,1,2*

KIT Mutation Analysis in Mast Cell Neoplasms: Recommendations of the European Competence Network on Mastocytosis

Michel Arock[1,2], Karl Sotlar[3], Cem Akin[4], Sigurd Broesby-Olsen[5], Gregor Hoermann[6], Luis Escribano[7], Thomas K. Kristensen[8], Hanneke C. Kluin-Nelemans[9], Olivier Hermine[10], Patrice Dubreuil[11,12,13,14], Wolfgang R. Sperr[15], Karin Hartmann[16], Jason Gotlib[17], Nicholas CP Cross[18], Torsten Haferlach[19], Andres Garcia-Montero[7], Alberto Orfao[7], Juliana Schwaab[20], Massimo Triggiani[21], Hans-Peter Horny[3], Dean D. Metcalfe[22], Andreas Reiter[20], and Peter Valent[15]

Flow cytometry: Objectives

- Retrospective review of our flowcytometric mast cell analysis
- Quantification of mast cells in control population and patients with systemic mastocytosis
- Evaluation of MFI (mean fluorescence intensity) ratio to determine positivity and negativity of immuunphenotypic expression of CD markers.
- Calculation of sensitivity and specificity of CD2, CD25, CD30

Flow cytometry: Methods

- Instrument: Navios (Beckman Coulter)
- Acquisition software: Kaluza 1.5a

- RBC lysis :
  - lyse wash using Optilyse – 100 µl bone marrow
  - bulk lysis using NH_4Cl – 1 ml bone marrow

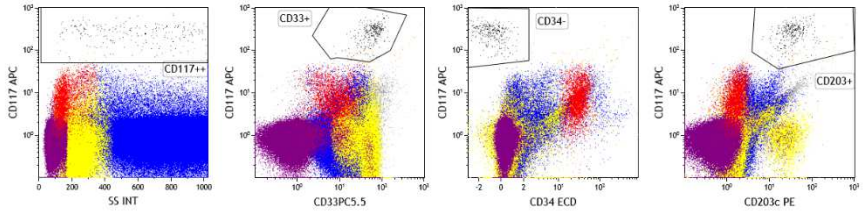
- ≥20 clustered CD117++ CD33+ CD34- CD203+ events: defined as mast cells
  - 40 control bone marrow samples
  - 18 bone marrow samples of systemic mastocytosis patiënts

Flow cytometry: Methods

8 color tube	FITC	PE	ECD	PC5.5	PC7	APC	AF700	KO
	CD30	CD203	CD34	CD33	CD2	CD117	CD25	CD45
Normal mast cells	-	+	-	+	-	++	-	+
Aberrant mast cells	+	+	-	+	+	++	+	+

- Low mast cell burden in most patiënts
- Sensitivity!
  - Total CD45+ events $5x10^5$
    - lowest quantifiable mast cell frequency 0.004% ((20 MC /500000 CD45+)*100)
  - Total CD45+ events $2x10^6$
    - lowest quantifiable mast cell frequency 0.001%

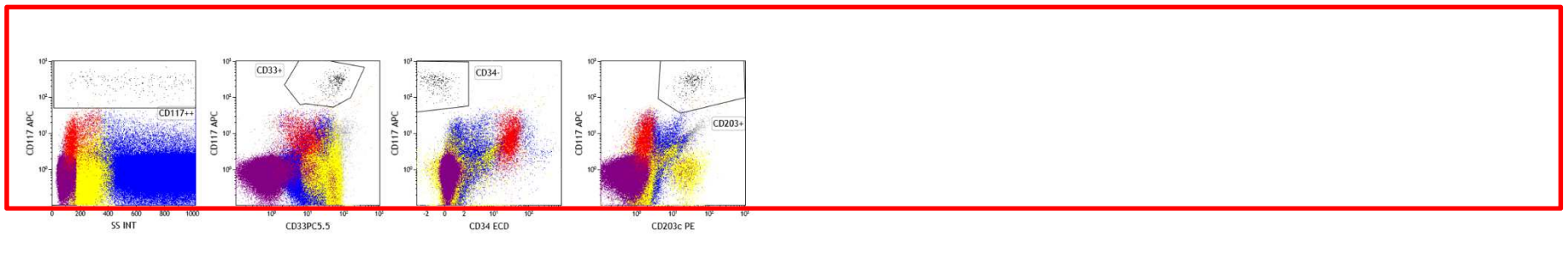
Quantification of mast cells



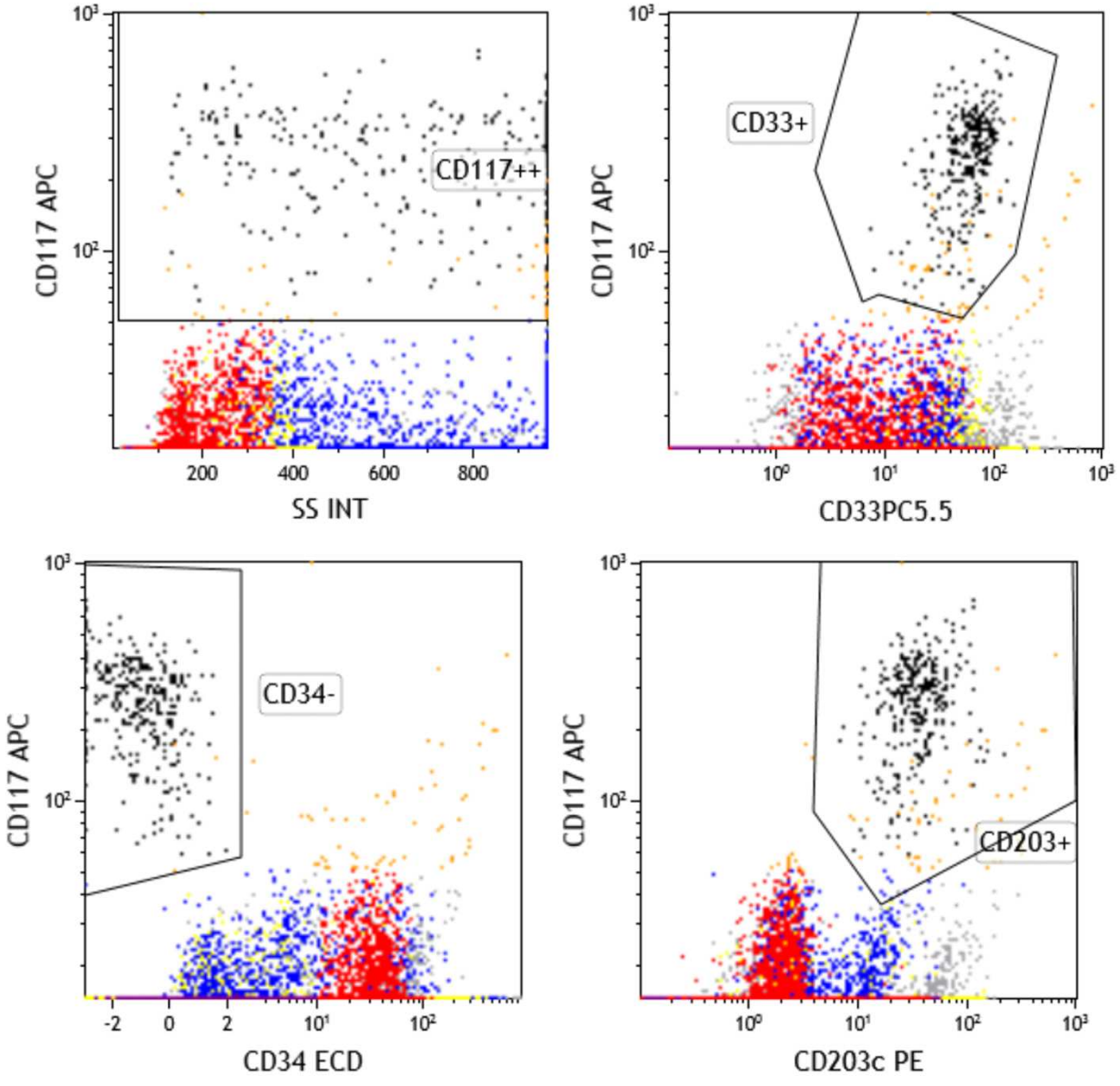
	Mast Cell range	
	UZA	Escribano et al 2006
Systemic Mastocytosis	0.014-2.4%	0.001-1.7%
control	0.0036-0.68%	0.001-0.09%

Color code
Mastcells
CD34+ blasts
CD33 monocytes
CD33+ granulocytes
Lymphocytes (CD45+ FS SS)

Zoom in CD117++



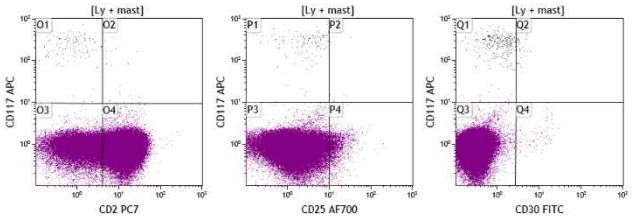
Zoom in CD117+ axis



122 events in CD117++ gate (black and orange) (0,04%)

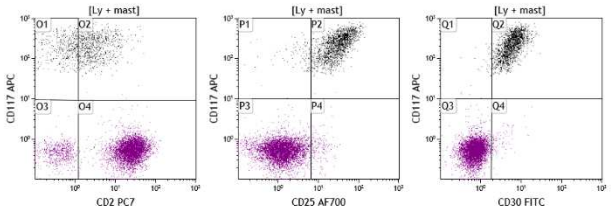
46 events in CD117+ CD33+ CD34- CD203+ gate (black) (0,015%)

Normal mast cells: CD2- CD25- CD30-

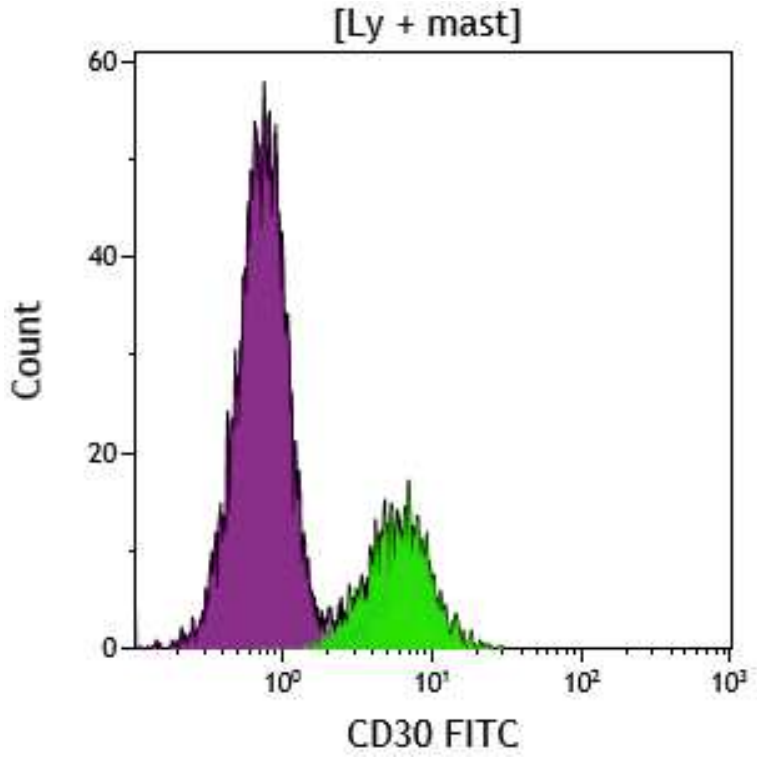


Gate: lymphocytes + mast cells
Lymphocytes negative control

Aberrant mast cells: CD2+ CD25+ CD30+



Mean Fluorescence Intensity (MFI) ratio



Criteria SKML

MFI ratio **>10**: marker positive

3.3 ≤ MFI ratio **≤ 10**: marker weak positive

MFI ratio **< 3.3**: marker negative

$$\text{MFI ratio} = \frac{\text{MFI}_{\text{mast cells}}}{\text{MFI}_{\text{lymphocytes}}}$$

Sensitivity/specificity of CD2, CD25 and CD30

MFI ratio >10			
	CD2	CD25	CD30
Spec	100%	100%	96%
Sens	72%	94%	61%

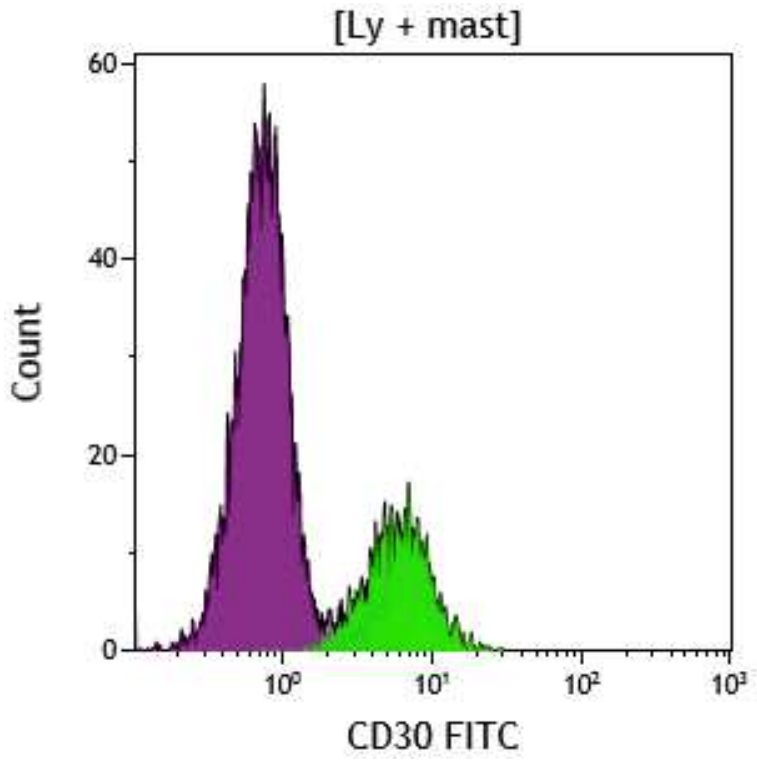
Good specificity:
control population CD2-, CD25-, CD30-

Sensitivity:
acceptable for CD25: 94 % of patiënts CD25+
only 72% and 61% of patiënts CD2+, CD30+

MFI ratio ≥ 3.3			
	CD2	CD25	CD30
Spec	88%	88%	54%
Sens	83%	100%	100%

Specificity:
lowest for CD30: 54% of normal controls CD30-

Sensitivity: very good for CD25 and CD30: all patiënts are positive



Criteria SKML

MFI ratio **>10**: marker positive

3.3 ≤ MFI ratio **≤ 10**: marker weak positive

MFI ratio **< 3.3**: marker negative

Calculation of cut-off for positivity after ROC-curve analysis **≥ 7.5**

$$\text{MFI ratio} = \frac{\text{MFI}_{\text{mast cells}}}{\text{MFI}_{\text{lymphocytes}}}$$

Sensitivity/specificity with own cutt-off

MFI ratio ≥7.5			
	CD2	CD25	CD30
Spec	100%	100%	92%
Sens	78%	94%	83%

Combined use	
Spec	96%
Sens	100%

Flow cytometry: conclusion

- Multiparametric immunophenotyping
- SM patients have a higher MC range in comparison to a control population
- MFI ratio is an objective analysis method for scoring aberrant markers as positive/negative
- In our data set a MFI ratio cut-off ≥ 7.5 shows the best performance
- CD30 is, beside CD2 and CD25, also a specific and sensitive marker for neoplastic mast cells and helpful in the diagnosis of systemic mastocytosis.

Molecular diagnostics

CKIT D816V mutation detection

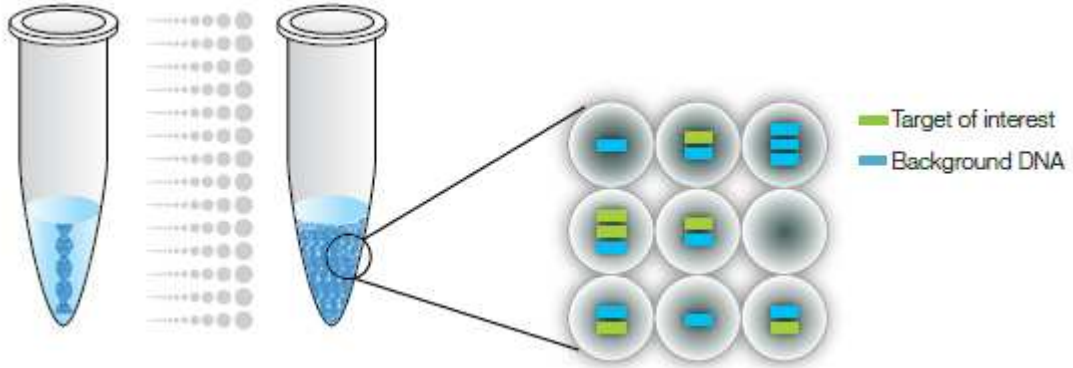
- Recommendations of the European Competence network on Mastocytosis (Arock et al 2015)
  - Low mast cell burden
  - Highly-sensitive KIT D816V detection
  - Initial screening in blood (in stead of bone marrow)
  - Quantitative follow-up of allele burden
- Kristensen et al J. Molec Diagn 2011 en Kristensen et al Am J. Hem 2014
  - Allele specific quantitative PCR
  - Sensitivity: 0,01-0,1%

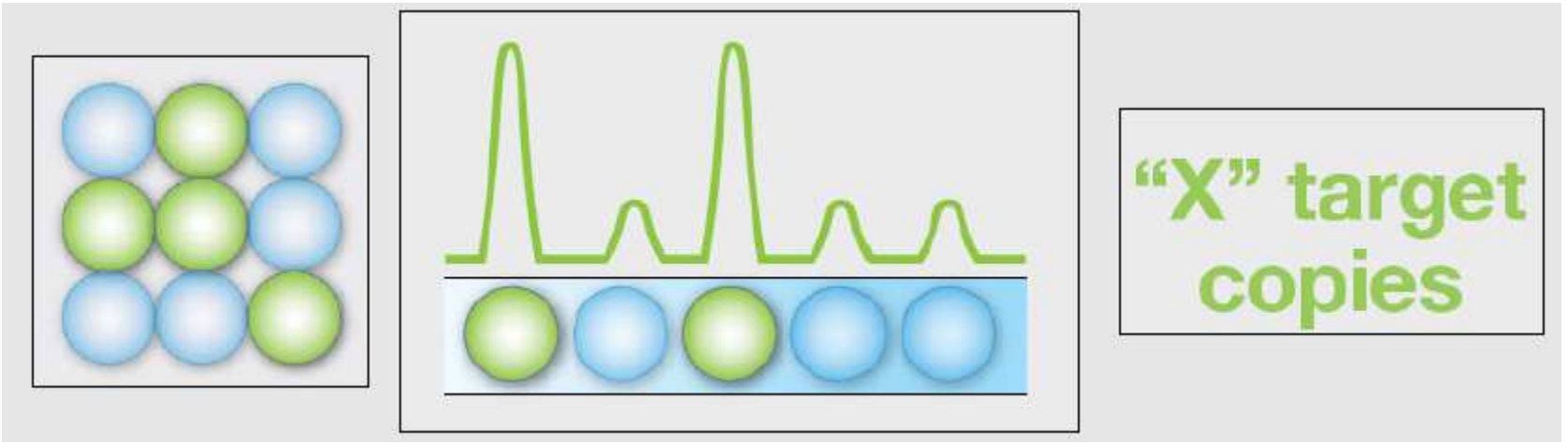
Digital droplet PCR

- sample is partitioned in 20000 droplets
- PCR amplification occurs in each individual droplet
- end-point data collection
  - not dependent on PCR efficiency
- no standard curve is needed for absolute quantification
  - counting the number of positive and negative droplets
  - Poisson correction on positive partitions
- high sensitivity and precision in detection and quantification of rare mutations

Digital droplet PCR

Sample is partitioned in 20000 droplets (Biorad QX200 droplet generator)





PCR amplification in each droplet

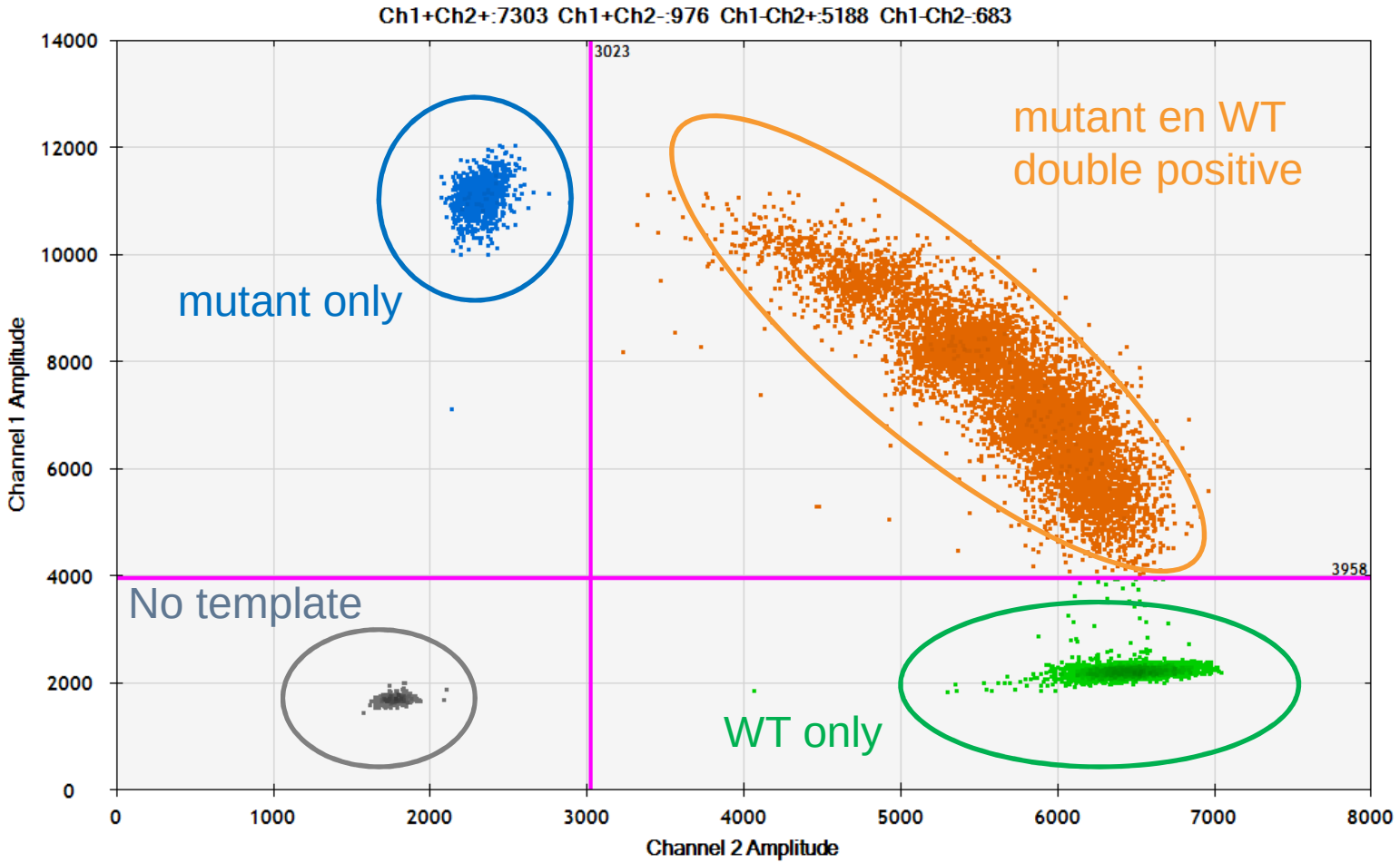
Droplet reader (Biorad QX200): two-color detection system (FAM/HEX)

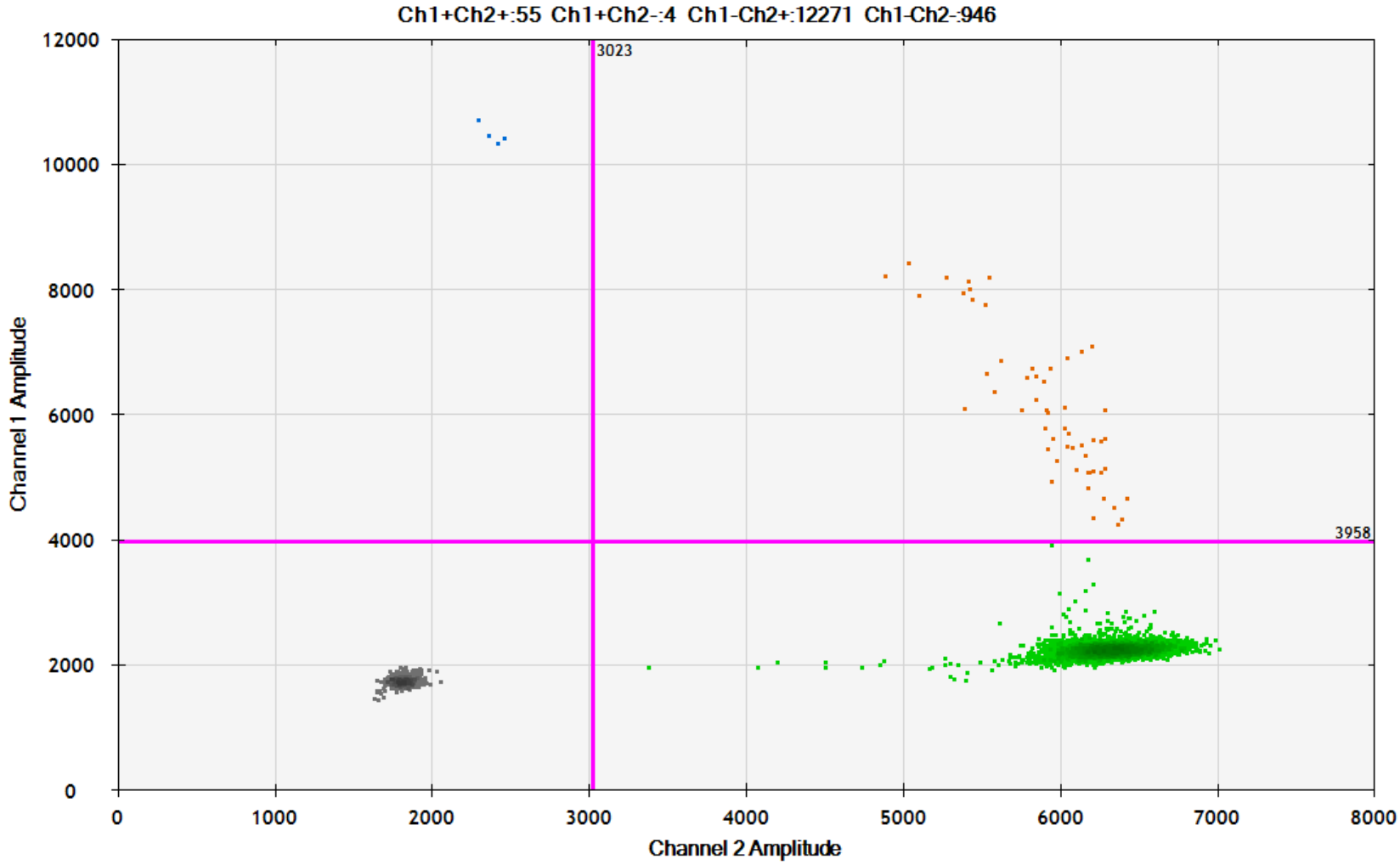
QuantaSoft: data analysis

Digital droplet PCR for KIT D816V mutation

- Bio-Rad Laboratories: rare mutation assays for cancer mutation
  - Single set of primers for mutated and wild type gene
  - Two competitive probes
    - WT allele in HEX
    - Mutant allel in FAM
- Four droplet clusters in 2-D scatterplot
  1. double negative droplets containing no targeted DNA templates
  2. Wild type-only droplets
  3. Mutant-only droplets
  4. Double positive droplets containing wild type and mutant DNA templates
- Concentrations are calculated based on the number of droplets in each cluster

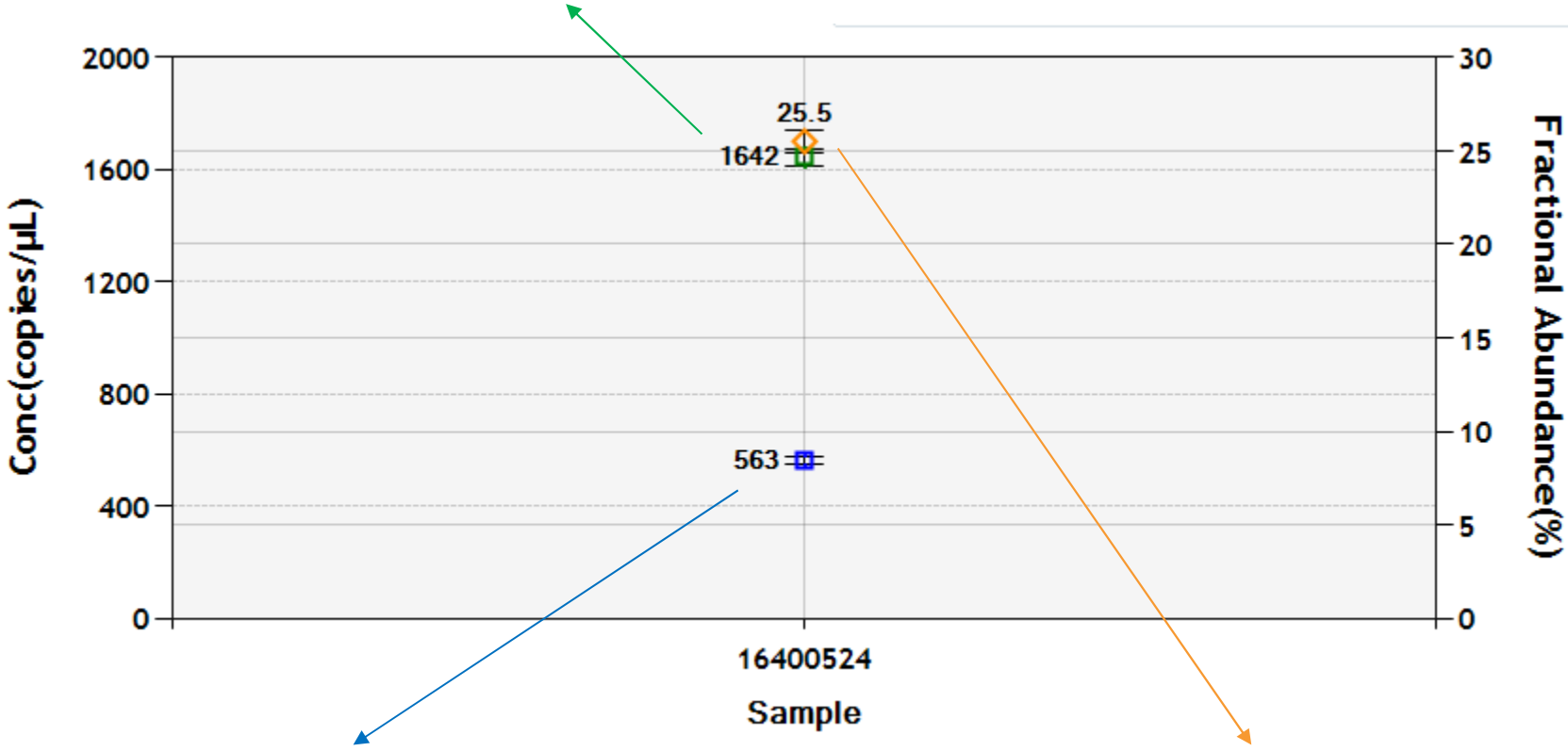
Location of droplets in 2-D scatterplot




Ch1+Ch2+:55 Ch1+Ch2-:4 Ch1-Ch2+:12271 Ch1-Ch2-:946
3023
3958
Channel 1 Amplitude
Channel 2 Amplitude
0
2000
4000
6000
8000
10000
12000
0
1000
2000
3000
4000
5000
6000
7000
8000

Concentration

Wild type template concentration (copies/µl)



Mutant template concentration (copies/µl)

% mutant

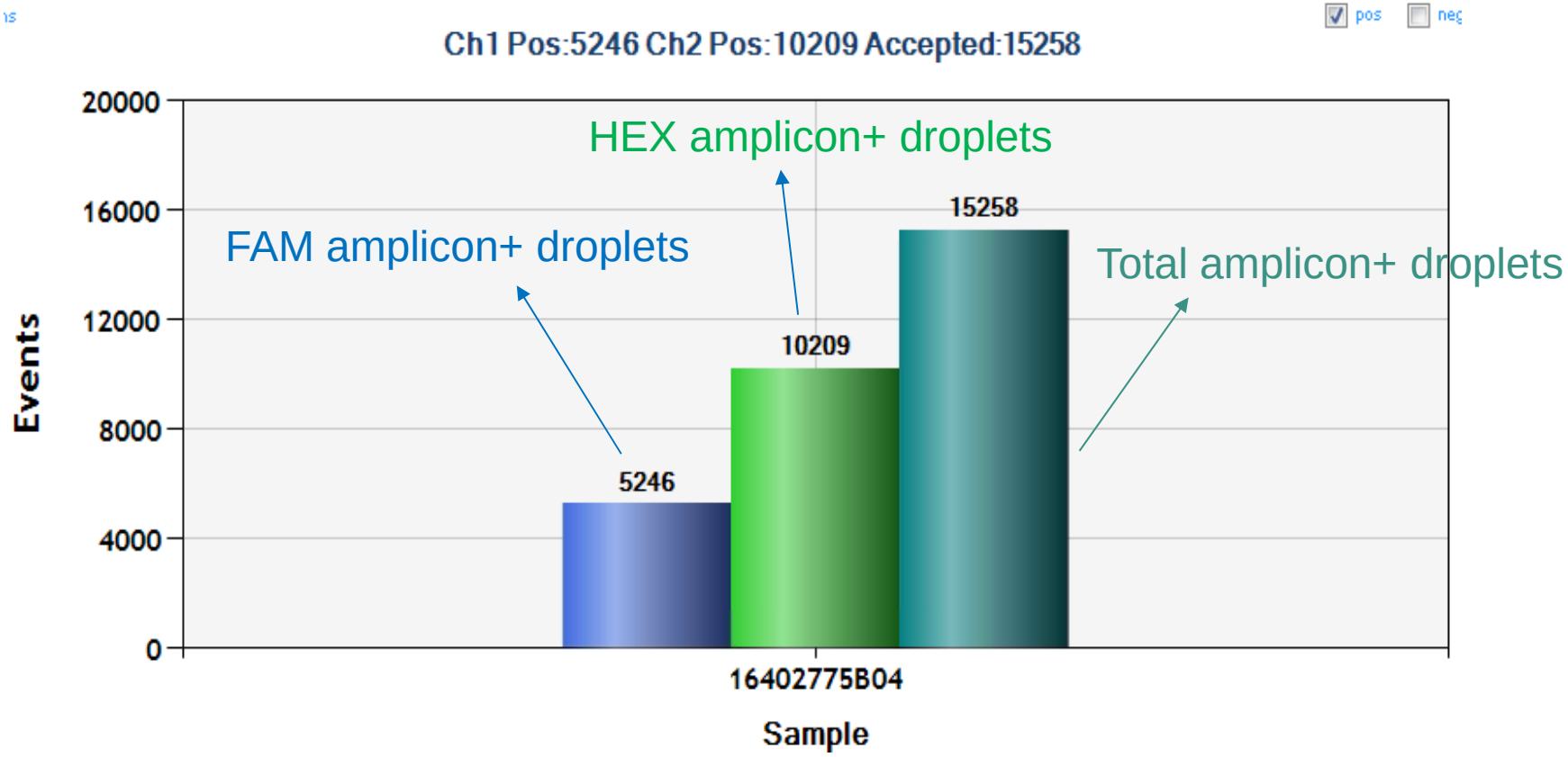
Validation ddPCR

- Repeatability
- Reproducibility
- Linearity and sensitivity
  - 10-fold dilution series of DNA derived from patient material/Horizon into wild-type DNA
- Accuracy
  - Patients fulfilling WHO criteria (other than presence of CKIT mutation) for systemic mastocytosis
- Specificity
  - Control population

Results

- Repeatability
  - Triplicate experiments
    - 9 KIT D816V positive samples, different allele burden
    - %CV between 0,4-5,4%
- Reproducibility
  - Two different patient samples
    - average of 38% and 0.19% mutation-positive cells
    - repeated in ten ddPCR runs
    - %CV of 1.1% en 7.6%
    - No more than 1.3 fold differences between minimum and maximum measured % mutation-positive cells

Criteria: # positive droplets



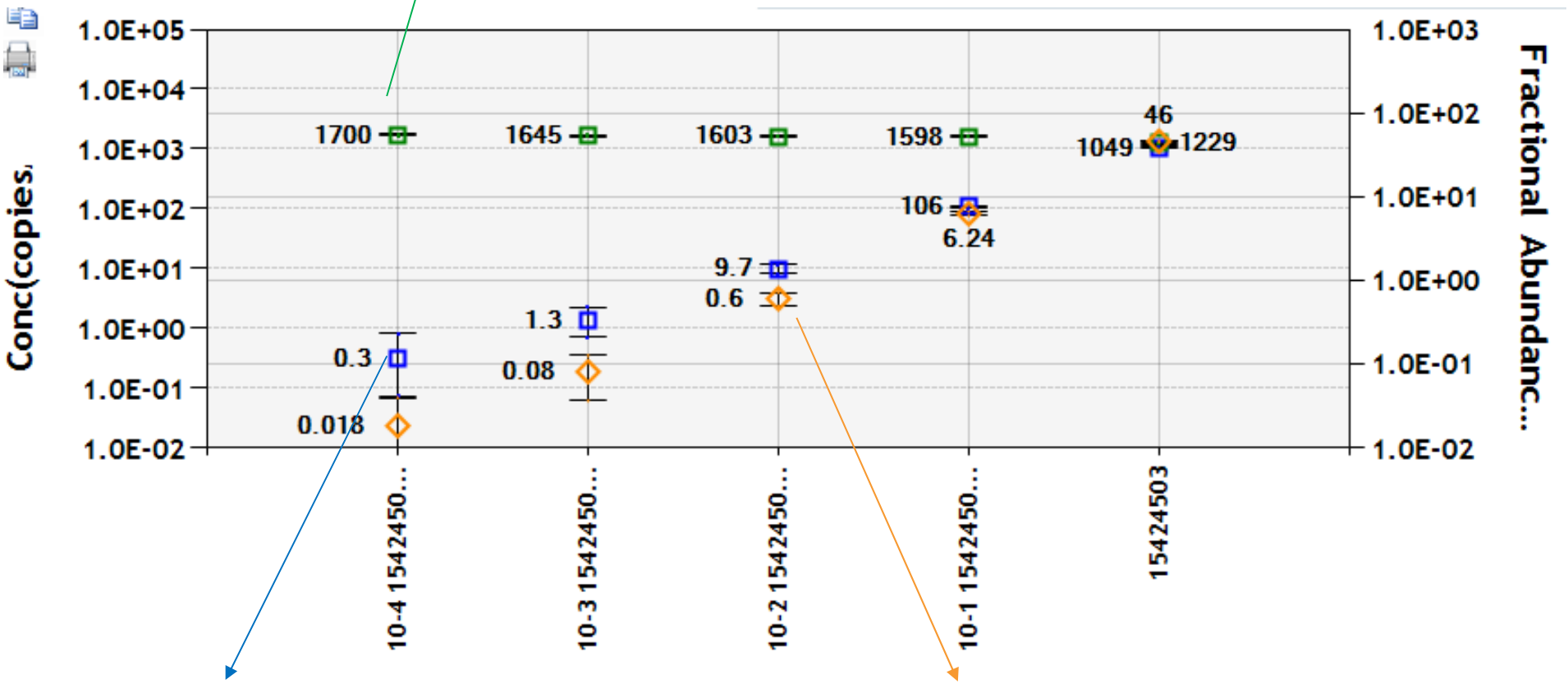
>10000 amplicon+ droplets in standard experiment

Lower # droplets in old samples/bad DNA quality

>500 amplicon+ droplets: % CV test remains reliable

Linearity and sensitivity: 10 fold dilution

Wild type template concentration (copies/µl)



Mutant template concentration (copies/µl)

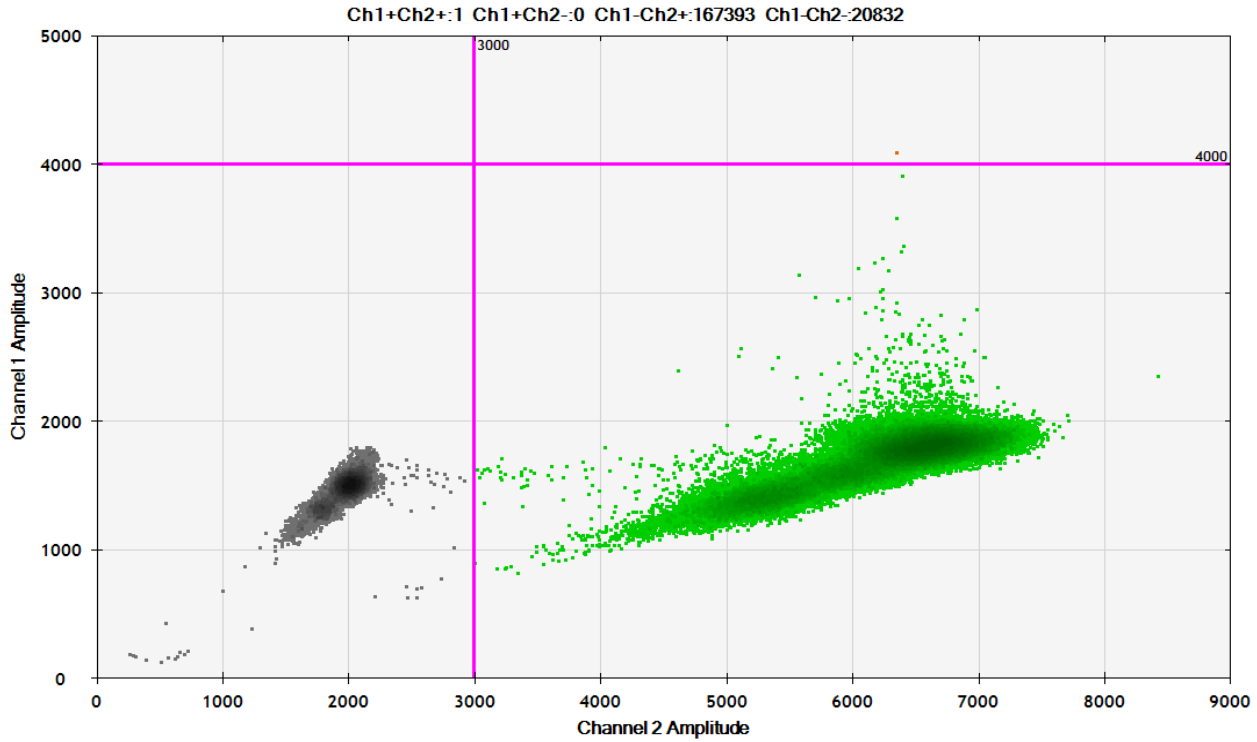
% mutant

Sample specific sensitivity

- Quantasoft result:
  - max 5000 copies/µl
  - 300 ng starting DNA in 1 well)
- Fixed reaction volume 20 µl
- 5000 copies/µl *20 µl= 100000 genome equivalents
- Limit of detection: at least 3 mutation positive droplets
- Sample specific sensitivity = (3/100000)*100=0,003%
  - Merged triplicate experiment sensitivity = 0,001%
- Routine practice: 1 sample: triplicate, 200 ng/well, 0,002% sensitivity
- Negative samples are repeated when 0,01% is not achieved

Specificity

- CKIT ddPCR negative in
  - 10 healthy individuals
  - 3 reactive bone marrows



Accurarcy

- CKIT positive in
  - 20 samples
    - 5 blood samples
    - 15 bone marrow samples
  - 14 patiënts
    - 13 indolent systemic mastocytosis
    - 1 systemic mastocytosis with associated CMML
  - Allelic burden
    - Blood between 0,011-30%
    - Bone marrow 0,011-47%
  - Implemented in routine diagnostics january 2016

Molecular diagnostics: conclusion

- KIT D816 V detection using digital droplet
  - Good reproducibility
  - Quantitative method
  - Sensitive (<0,01%)
- Usefull to screen peripheral blood of suspected patiënts

Mutations other than KIT D816V

- KIT D816H, KIT D816Y, …
- Additional somatic mutations apart from KIT mutation (in advanced systemic mastocytosis)
  - TET2, SRSF2, ASXL1, RUNX1, JAK2, RAS
  - Adverse prognosis
  - Should be considered in upcoming prognostic scoring systems
- Next generation sequencing

UZA team

Klinische biologie-Labo Hematologie

Inge De Backer
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Stéphanie Fournier
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Daphné Lathouwers
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Marie-Berthe Maes
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Anatoom Pathologie

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