Mouse Monoclonal Antibody (CAL2) Against All *CALRETICULIN* (*CALR*) Mutations

Technical Note **CALRmut IHC**

Procedures:
I. Manual stain
II. Automated: DAKO Omnis
III. Formalin- / B5- Fixative recommendations

Catalog No.:	DIA-CAL-100 (100µl) DIA-CAL-250 (250µl)
Clone:	CAL2
Isotype:	Mouse IgG2a
Physical State:	Lyophilized powder
Reconstitution:	DIA-CAL-100 restore to 100µl DIA-CAL-250 restore to 250µl Reconstitute with sterile distilled water by gentle shaking for 10 minutes
Dilution	1:50-1:100

I. Manual IHC stain - Pressure cooker

1. Deparaffinization

a.	4x Xylol-bath	5 min each
b.	1x EtOH 100%	30 sec
c.	1x EtOH 100%	5 min
d.	1x EtOH 90%	5 min
e.	1x EtOH 70%	5 min
	-> Keep in cuvette with a.dest.	

2. Heat-induced epitope retrieval:

Pressure cooker cuvette with Citrate buffer 10mMol pH6

a.	Incubation	20 min
b.	Cool at RT	20 min

3. Staining:

a.	Application of Primary Antibody:	
	DIA-CAL, Dilution 1:100, 100 µl	
	Incubation time	30 min
	-> Wash in PBS buffer	30 sec
b.	Application of Secondary Antibody:	
	Dako REAL™ Detection System, Alk.Phosphatase/RED,	
	- Dako REAL™ Link, Biotinylated Sec. Ab (AB2)	30 min
	-> Wash in PBS buffer	30 sec
	- Dako REAL™ Streptavidin Alk.Phosph. (AP)	30 min
	-> Wash in PBS buffer	30 sec
c.	Application of Chromogen:	
	Dako REAL™ Chromogen Red	
	- 100 µl RED	16 min
	-> Wash with water	1,5 min

4. Nuclear Counterstain:

a.	100 µl Haemalum	7 min
	-> Wash with water	1,5 min

5. Cover: In Xylol

ONCOdianova GmbH
Warburgstrasse 45
D-20354 Hamburg
Germany

URL: www.oncodianova.com
Email: info@oncoddianova.com
Phone: +49 (0)40 – 45067 – 0
Fax +49 (0)40 – 45067 – 490

II. Automated IHC stain – Dako Omnis

Platform:	Dako Omnis
Kit:	EnVision FLEX HRP Magenta, High pH, Code GV900
Heat-induced epitope retrieval:	EnVision FLEX Target Retrieval Solution, High pH, Tris/EDTA buffer, pH 9 (DM 848), 97 °C, 40 min
Block	EnVision FLEX Peroxidase-Blocking Reagent (DM841), 3min
Secondary antibody (linker)	EnVision FLEX+ Mouse LINKER, 10 min
Polymer	EnVision FLEX /HRP (DM842), 20 min
Chromogen	EnVision FLEX HRP Magenta Chromogen (DM857), 5min
Wash buffer:	Dako Omninis, Code GC 807, 10 cycles á 2 min
Antibody diluent:	Dako REAL Antibody Diluent (Code S2022)
Primary antibody:	DIA-CAL (clone Cal2) 1:50, 200µl
Counterstain:	Hematoxylin (Dako Omninis, Code GC808), 7 min

III. Recommendations for Formalin & B5-fixed bone marrow biopsies

Alkaline Phosphatase (AP) based detection (with Biotin-Avidin amplification)

1. Formalin-fixed and EDTA-decalcified bone marrow biopsies:
Protocol for Biotin-Avidin-amplified Alkaline Phosphatase (AP) based detection

a. Maturation of paraffin sections in incubator (overnight at 39°C or 1h at 56°C)
b. Deparaffinization
c. Ag retrieval: 10’ cooking in pressure cooker in citrate buffer (10.5g / 5l), pH 6.0 (it may be longer for acid decalcified tissue)
4. Cool by rinsing with water
5. Wash with distilled water and subsequently with wash buffer
6. Incubation with primary Ab at RT: 30’manual stain / 45‘ automatic stainer
7. Wash with wash buffer
8. Incubate with biotinylated secondary Ab at RT for 15’
9. Wash with wash buffer
10. Incubate with Streptavidin Alkaline Phosphatase at RT for 15’
11. Wash with wash buffer
12. Incubate with substrate (Substrate buffer + chromogens + Levamisole), 20’ RT
13. Wash with wash buffer
14. Hematoxylin staining
15. Wash with Isopropanol x2
16. Wash with Xylol x2
17. Cover with cover glass

2. B5-fixed and EDTA-decalcified bone marrow biopsies:
Antigen retrieval
with EDTA-buffer pH8.0, 10min pressure cooker or 30-60min microwave at 98-100°C

3. B5-fixed and acid-decalcified bone marrow biopsies:
Antigen retrieval
with EDTA-buffer pH8.0, 10min pressure cooker or 30-60min microwave at 98-100°C

Results: **Formalin-fixed BM:**
Simple and fast analysis by a specific and intense staining of megakaryocytes and a variable amount of cells with small nucleus.
B5-fixed BM:
Simple and fast analysis by a specific and intense staining of megakaryocytes. Only include megakaryocytes for analysis. Sometimes weak non-specific staining of granulopoietic or erythropoietic cells.

ONCOdianova GmbH
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D-20354 Hamburg
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Fax +49 (0)40 – 45067 – 490