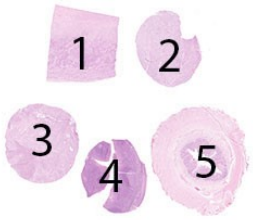
Assessment Run 75 2025

Podoplanin (Podop)

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for Podop, identifying seminomas in the characterization of tumours of unknown origin and differentiation between malignant mesotheliomas and non-small cell lung carcinomas (NSCLCs). Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for Podop (see below).

Material
The slide to be stained for Podop comprised:

1. Mesothelioma, 2. Seminoma, 3. NSCLC, 4. Tonsil, 5. Appendix



All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a Podop staining as optimal included:

- A strong, distinct predominantly cytoplasmic staining reaction of the lymphatic endothelial cells in all tissues.
- A weak to moderate, distinct predominantly cytoplasmic staining reaction of the Schwann cells of the muscularis propria in the appendix.
- A moderate to strong, distinct predominantly cytoplasmic staining reaction of the follicular dendritic network and the basal squamous epithelial cells in the tonsil, tissue core no. 4.
- A moderate to strong, distinct predominantly membranous staining reaction of the neoplastic cells in the seminoma, tissue core no. 2, and mesothelioma, tissue core no. 1.
- A negative staining reaction of the neoplastic cells of the NSCLC, tissue core no. 4, and epithelial cells in appendix and mature squamous epithelial cells in tonsil.

KEY POINTS FOR PODOP IMMUNOASSAYS
- The mAb clone **D2-40** was most widely used antibody. Used by 98% participants.
- An aberrant cytoplasmic staining of goblet cells in appendix was seen on platforms (Autostainer and Bond) with inefficient washing steps.
- The Ventana/Roche RTU **760-4395** system based on mAb clone D2-40 needs optimization to perform optimal.

Participation

Number of laboratories registered for Podop, run 75	409
Number of laboratories returning slides	373 (91%)

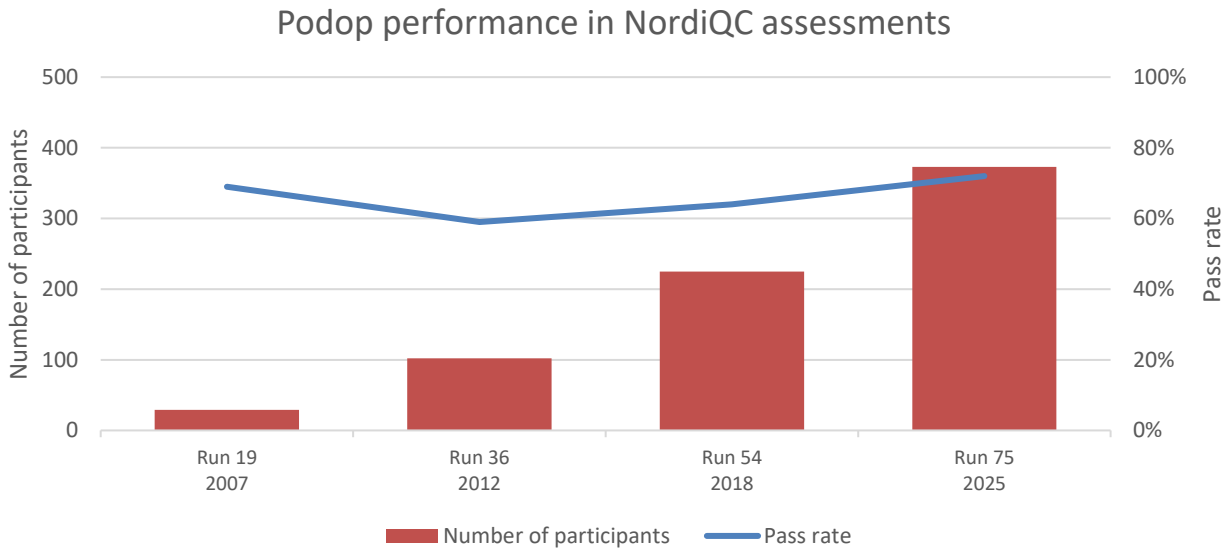
At the date of assessment, 91% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

Results
373 laboratories participated in this assessment. 268 (72%) of these achieved a sufficient mark (optimal or good) - see Table 1a (page 2). Tables 1b and 1c summarizes the antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining were:
- Use of less sensitive detection systems
- Too high concentration of the primary Ab
- Insufficient HIER
- Use of less successful Abs

Performance history
This was the fourth NordiQC assessment of Podop (see Graph 1). The number of participants increased from 225 laboratories in 2018 to 373 laboratories in the current assessment. Despite this 65% increase in participating laboratories, the pass rate improved slightly to the highest pass rate achieved of 72% compared to 64% in 2018.

Graph 1. **Proportion of sufficient results for Podop in the four NordiQC runs performed**



Controls
Virtually all lymphatic endothelial cells, basal squamous epithelial cells and follicular dendritic network must show a moderate to strong predominantly cytoplasmic staining reaction in tonsil.
In appendix, an at least moderate cytoplasmic staining of the Schwann cells in the muscularis propria of the appendix must be seen. In addition, endothelial cells of lymphatic vessels should be strongly stained, whereas no staining reaction should be seen in endothelial cells of the blood vessels and in the columnar epithelial cells and goblet cells of the mucosa.

Conclusion
Optimal staining results could be obtained with the mouse monoclonal Ab (mAb) clones **D2-40** and **MX025.** Virtually all laboratories (365 of 373) used mAb clone D2-40. Efficient HIER, preferably in alkaline buffer, use of a sensitive polymer/multimer detection system and careful calibration of the primary Ab were the most important prerequisites for an optimal staining result. Although Podop is a challenging marker, the concentrated format of the mAb clone D2-40 provided optimal staining results on all four main stainer platforms – Dako Autostainer, Dako Omnis, Leica Bond and Ventana BenchMark.
In general, the performance of the Ready-To-Use (RTU) systems was superior to the laboratory developed (LD) assays using the Abs in concentrated format. However, a significant difference in the performance and pass rates was observed within the RTU systems. The proportion of sufficient staining results following vendor recommended protocol settings was only 44% for the Ventana/Roche 760-4395 RTU-system compared to 100% for the Dako/Agilent IR072 RTU system. The IR072 was mitigated to the Dako Omnis platform by 65 laboratories, with a pass rate of 88%.

Table 1a. **Overall results for Podop, run 75**

	n	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
Concentrated antibodies	87	25	26	28	8	59%	29%
Ready-To-Use antibodies	286	98	119	66	3	76%	34%
Total	373	123	145	94	11		
Proportion		33%	39%	25%	3%	72%	

1) Proportion of sufficient results (optimal or good).
2) Proportion of optimal results.

Table 1b. **Concentrated antibodies and assessment marks for Podop, run 75**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **4D5aE5E6**	1	ThermoFisher	0	0	0	1	-	-
mAb clone **D2-40**	1	Bio Legend	0	0	1	0	-	-
	4	BioCare Medical	1	1	1	1	-	-
	1	BioSB	0	0	0	1	-	-
	10	Cell Marque	4	4	2	0	80%	40%
	60	Dako/Agilent	20	18	19	3	63%	33%
	1	Diagnostic BioSystems	0	1	0	0	-	-
	1	Origene Technologies	0	0	1	0	-	-
	4	Zytomed Systems	0	1	1	2	-	-
mAb clone **GM27**	1	GeneBioSolution	0	0	1	0	-	-
mAb clone **IHC650**	1	GenomeMe	0	1	0	0	-	-
rmAb clone **QR048**	1	Quartett	0	0	1	0	-	-
rmAb clone **ZR442**	1	Zeta Corporation	0	0	1	0	-	-
Total	87		25	26	28	8		
Proportion			29%	30%	32%	9%	59%	

1) Proportion of sufficient results (optimal or good),
2) Proportion of sufficient results with optimal protocol settings only, see below.

Table 1c. **Ready-to-use** a**ntibodies and assessment marks for Podop, run 75**

Ready-To-Use antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **D2-40 PM266**	3	BioCare Medical	0	2	1	0	**-**	**-**
mAb clone **D2-40 322M**	22	Cell Marque	7	8	7	0	68%	32%
mAb clone **D2-40 IR072 (VRPS)[3]**	16	Dako/Agilent	4	12	0	0	100%	25%
mAb clone **D2-40 IR072 (LMPS)[4]**	82	Dako/Agilent	33	34	13	2	82%	40%
mAb clone **D2-40 MAD-000402QD**	5	Master Diagnostica	1	1	2	1	40%	20%
mAb clone **D2-40 8515-C010**	3	Sakura Finetek	3	0	0	0	**-**	**-**
mAb clone **D2-40 760-4395 (VRPS)[3]**	48	Ventana/Roche	0	21	27	0	44%	0%
mAb clone **D2-40 760-4395 (LMPS)[4]**	100	Ventana/Roche	46	40	14	0	86%	46%
mAb clone **D2-40 BSB6065**	1	BioSB	1	0	0	0	-	-
mAb clone **D2-40 CDM-0010**	1	Celnovte	1	0	0	0	-	-
mAb clone **D2-40 GM361902**	1	Gene Tech	0	1	0	0	-	-
mAb clone **D2-40 MAB-0567**	1	Fuzhou Maixin	1	0	0	0	-	-
mAb clone **MX025 MAB-0714**	1	Fuzhou Maixin	1	0	0	0	-	-
rmAb clone **QR048 P-P016**	1	Quartett	0	0	1	0	-	-
Ab clone **DY49959 4912042**	1	Dakewe	0	0	1	0	-	-
Total	286		98	119	66	3		
Proportion			34%	42%	23%	1%	76%	

1) Proportion of sufficient results (optimal or good). (≥5 assessed protocols).
2) Proportion of optimal results (OR).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s), non-validated semi/fully automatic systems or used manually (≥5 assessed protocols).

Detailed analysis of Podop, Run 75
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **D2-40**: Protocols with optimal results were all based on Heat Induced Epitope Retrieval (HIER) using Cell Conditioning Solution 1 (CC1, Ventana/Roche) (10/35)*, Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (7/27), Target Retrieval Solution (TRS) high pH (Dako/Agilent) (6/12), Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (1/4), or unknown (1/1). The mAb was diluted in the range of 1:10-1:150 in combination with a 3-step detection system. Using these protocol settings, 48 of 70 (69%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

Table 2. **Proportion of optimal results for Podop for the most commonly used antibodies as concentrates on the 4 main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer[1]**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark[2]**		**Leica Biosystems Bond[3]**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **D2-40**	2/2**	0/1	4/9 (44%)	-	10/28 (36%)	-	7/26 (27%)	1/4

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective systems.
** (number of optimal results/number of laboratories using this buffer)
1) Autostainer Classical, Link 48+.
2) BenchMark GX, Ultra, Ultra plus
3) Bond III, MAX, Prime

Ready-To-Use antibodies and corresponding systems
mAb clone **D2-40**, product no. **IR072**, Dako/Agilent, Autostainer+/Autostainer Link 48+:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 20 min. at 95-99°C), 15-20 min. incubation of the primary Ab and EnVision FLEX (K8000/K8002) (2-step polymer system) as detection system. Using these protocol settings, 17 of 17 (100%) laboratories produced a sufficient staining result (optimal or good).
The product was used by 75 laboratories on a non-intended platform. These data are not included here.

mAb clone **D2-40**, product no. **760-4395**, Ventana/Roche, Ventana Benchmark GX/XT/Ultra/Ultra Plus:
Protocols with optimal results were typically based on HIER in CC1 (efficient heating time for 24-64 min. at 95-100°C), 24-48 min. incubation at 36°C of the primary Ab and 3-step multimer detection systems, UltraView (760-500) with amplification (760-080), OptiView (760-700) or OptiView with tyramide signal amplification (760-099 / 860-099). Using these protocol settings, 76 of 80 (95%) laboratories produced sufficient staining results.
The product was used by 3 laboratories on a non-intended platform. These data are not included here.

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the intended IHC stainer device are included.

Table 3. **Proportion of sufficient and optimal results for Podop for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS mAb clone **D2-40, IR072**	100% (16/16)	25% (4/16)	57% (4/7)	14% (1/7)
VMS GX, XT, Ultra, Ultra plus mAb clone **D2-40, 760-4395**	44% (21/48)	0% (0/48)	86% (83/97)	45% (44/97)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered >25%, detection kit – only protocols performed on the specified vendor IHC stainer integrated.

Comments
In this assessment and in concordance with the previous NordiQC assessments of Podop, the prevalent features of an insufficient staining were either a generally too weak or false negative staining reaction of the cells and structures expected to be demonstrated and seen in 84% of the insufficient results (87 of 104). A poor signal-to-noise ratio or excessive background was seen in 13% of the insufficient results (14 of 104) and false positive staining was seen in slides from 3 laboratories (3%). Virtually all laboratories were able to demonstrate Podop in the neoplastic cells of the seminoma and in the majority of the lymphatic endothelial cells in all tissue cores, whereas the demonstration of Podop in the mesothelioma, follicular dendritic network

in the tonsil and in Schwann cells of the appendix was more challenging and required an optimally calibrated protocol.

23% (87 of 373) of the laboratories used Abs in a concentrated format within LD-assays for Podop (see Table 1b). Virtually all laboratories used **mAb clone D2-40** (82 of 87) and optimal staining results could only be obtained with this clone. Optimal staining results could be obtained on all major stainer platforms (Dako Autostainer, Dako Omnis, Ventana BenchMark and Leica Bond) – see Table 2. Irrespective of the stainer platform used, careful calibration of the titre and efficient HIER at high pH and a 3-step detection system were the main protocol prerequisites for optimal results. Only six laboratories used a 2-step detection system with a 33% pass rate, no optimal results.

77% (286 of 373) of the laboratories used Abs in RTU formats (see Table 1c).
The **Ventana/Roche RTU system 760-4395** based on mAb clone D2-40 was the most widely used RTU system and applied by 145 laboratories on the intended BenchMark platform. 33% (48 of 145) of the laboratories used the 760-4395 RTU system in compliance with the vendor recommended protocol settings, of which 48% (21 of 48) laboratories achieved sufficient staining results, no optimal.
For the laboratories modifying the protocols, the proportion of sufficient and optimal staining results improved to 86% (83 of 97) and 45% (44 of 97), respectively (see Table 3). Typically, if the vendor recommended 2-step multimer detection system (UltraView) was substituted with sensitive 3-step multimer detection system (OptiView) with or without OptiView Amplification, an optimal staining reaction could be achieved. These data are similar to results obtained in run 54 (2018) and still indicate a need for recalibration of the 760-4395 RTU system for the Ventana BenchMark platform and update of the vendor recommended protocol.

The mAb clone D2-40 based **IR072 (Dako/Agilent)**, intended for use on the Dako Autostainer System, gave a 100% pass rate (16 of 16) if following the vendor recommended protocol settings, 25% optimal (n=4) – see Table 3. Seven laboratories modified the protocol on Dako Autostainer, giving a 57% pass rate (4 of 7), 14% optimal (n=1). Typically, minor adjustments in HIER time and incubation time of primary Ab were seen. An aberrant cytoplasmic staining of goblet cells in appendix was seen in 65% (15 of 23) of the protocols on Dako Autostainer. This staining pattern was also seen in the previous run 54 (2018), where also the lung carcinoma, expected to be negative, became false positive in 57% of the slides (8 of 14). A well-known observation on the Dako Autostainer platform is that the washing time in buffer (after primary Ab, link-Ab and polymer) varies with the number of slides stained in a given run. Running only a few slides typically results in short washing times, whereas a full run of 48 slides results in prolonged washing times (in total at least 10-15 min. longer). In theory, potential low affinity binding (cross reaction) to substances in goblet cells would be removed by prolonged and effective washing conditions. This theory is supported by the observation that when the IR072 RTU format was mitigated to Dako Omnis - known for its efficient "high temperature" (32°C) washing - and applied within an LD-assay no false positive staining reaction was seen. The overall pass rate for the Dako Omnis IR072 based LD-assays was 88% (57 of 65) with 45% (29 of 65) being optimal. All laboratories using the RTU format with FLEX+ as detection system and as such adding mouse linker obtained an optimal result (18 of 18), compared to 23% (11 of 47) using the 2-step detection system EnVision FLEX as recommended by Dako/Agilent for the intended platform, Dako Autostainer Link.

The obtained relative low pass rate of Podop seems to be influenced by lack of optimally calibrated RTU systems for the main IHC platforms and system providers. Dako/Agilent has a well performing RTU system for Autostainer Link, but at present not available for their most popular system, Dako Omnis. No RTU system is at present available from Leica Biosystems and the Ventana/Roche RTU system needs a protocol update as the performance using the recommended settings was shown to be inferior to the level expected.

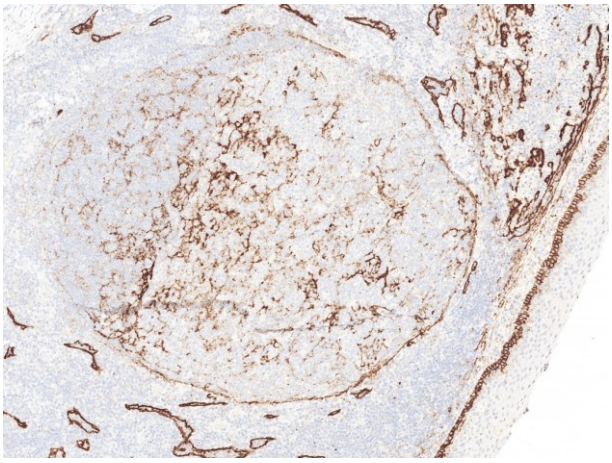


Fig. 1a
Optimal Podop staining reaction of the tonsil using a 760-4395 RTU (mAb clone D2-40) based LD-assay on the BenchMark Ultra. The vendor recommended protocol is modified, using the highly sensitive OptiView with tyramide signal amplification as detection system. A moderate to strong staining reaction of the lymphatic endothelial cells, follicular dendritic network and the basal squamous epithelial cells is seen.
Also compare with Figs. 2a-5a, same protocol.

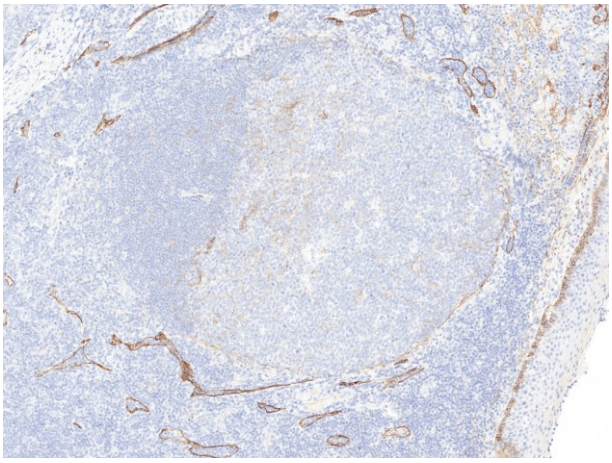


Fig. 1b
Insufficient Podop staining reaction of the tonsil using the mAb clone D2-40 based 760-4395 RTU-assay on the BenchMark Ultra. The vendor recommended protocol, based on the 2-step UltraView detection system, was followed. Weak to moderate staining of the lymphatic endothelial cells and the basal squamous epithelial cells is seen, whereas the follicular dendritic network is only faintly positive. The reduced analytical sensitivity was especially critical in the mesothelioma – see Fig. 3b.

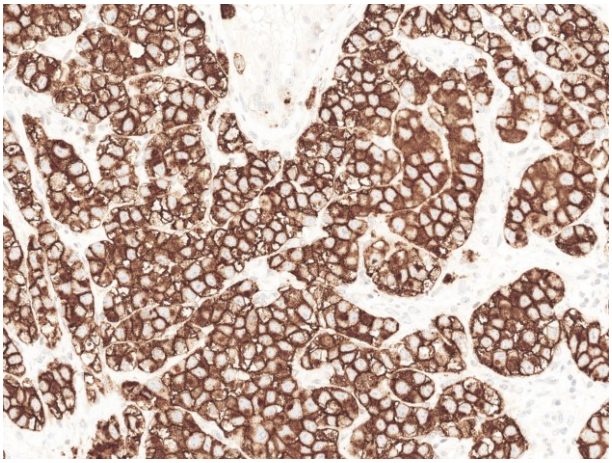


Fig. 2a
Optimal Podop staining reaction of the seminoma, tissue core no. 2, using same protocol as in Fig. 1a. A strong, distinct predominantly membranous staining reaction of the neoplastic cells is seen.

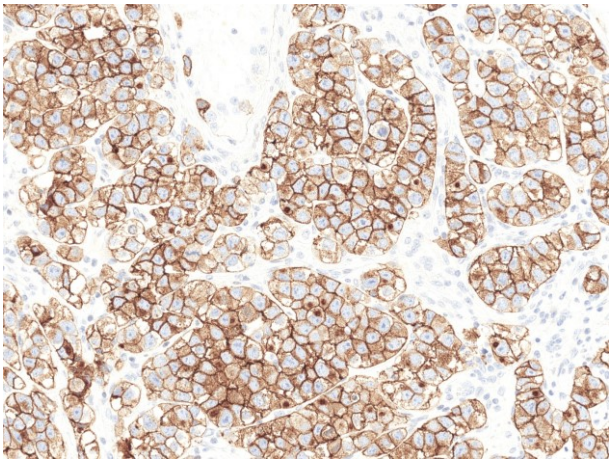


Fig. 2b
Podop staining reaction of the seminoma, tissue core no. 2, using same protocol as in Fig. 1b. A weak to moderate, predominantly membranous staining reaction of the neoplastic cells is seen.

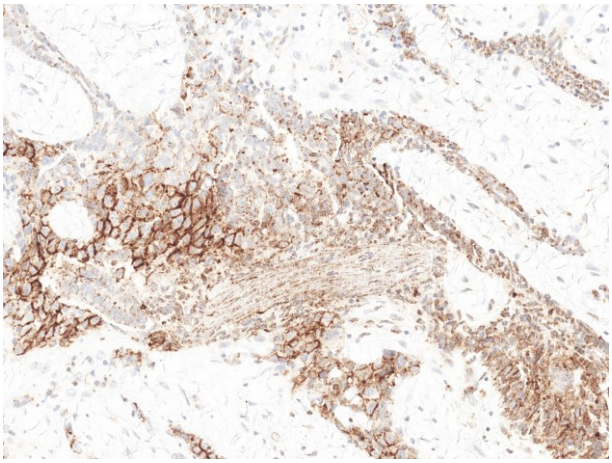


Fig. 3a
Optimal Podop staining reaction in the mesothelioma, tissue core no. 1, using same protocol as in Figs. 1a–2a. A moderate to strong, distinct predominantly membranous staining reaction of the neoplastic cells is seen. No background staining is seen, and a high signal-to-noise ratio is obtained.

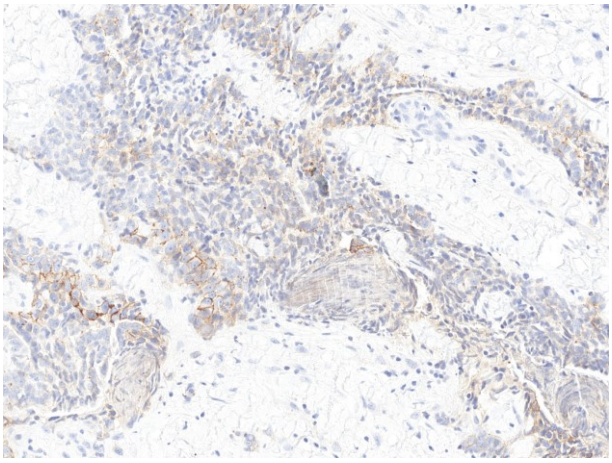


Fig. 3b
Insufficient Podop staining reaction in the mesothelioma, tissue core no. 1, using same protocol as in Figs. 1b–2b. Only a weak to moderate staining reaction is seen in scattered neoplastic cells significantly compromising the interpretation. Compare with Fig. 3a – same field.

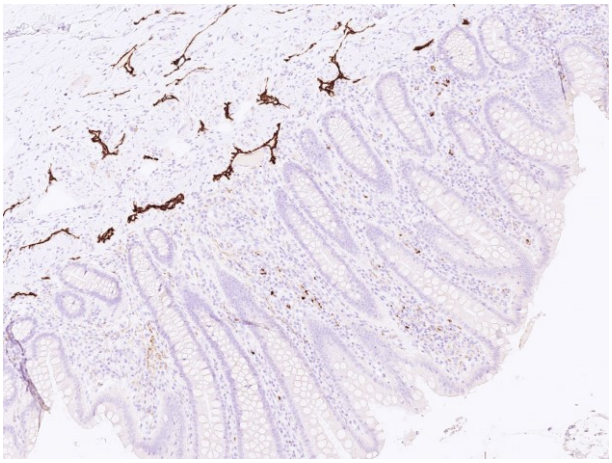


Fig. 4a
Optimal Podop staining reaction in appendix using same protocol as in Figs. 1a–3a. An expected strong staining of the lymphatic endothelial cells is seen whereas the low-level Podop expressing Schwann cells in the muscularis propria shows a moderate staining reaction.
The epithelial cells are all negative and in general a high signal-to-noise ratio is obtained.

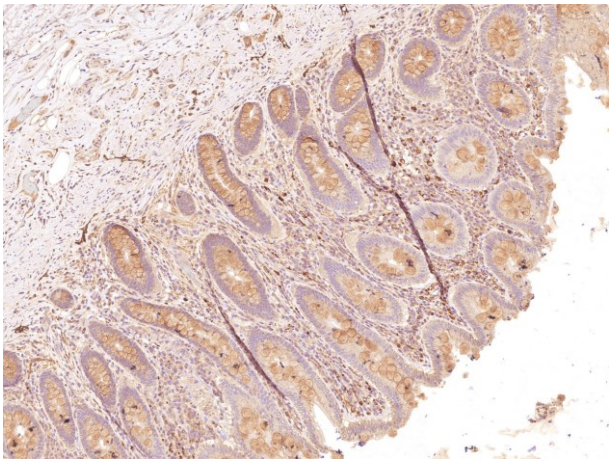


Fig. 4b
Insufficient Podop staining reaction in appendix using the mAb clone D2-40 on a Bond platform with HIER in BERS1. An aberrant cytoplasmic staining of goblet cells and a general poor signal-to-noise ratio is seen.

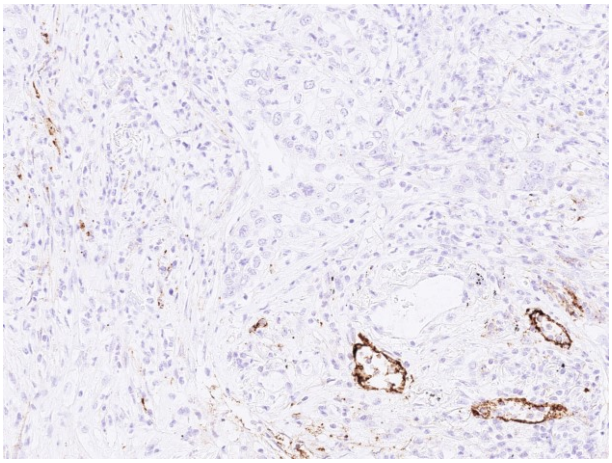


Fig. 5a
Optimal Podop staining reaction in the NSCLC using same protocol as in Figs. 1a–4a. An expected strong staining of the lymphatic endothelial cells is seen whereas the neoplastic cells are negative.

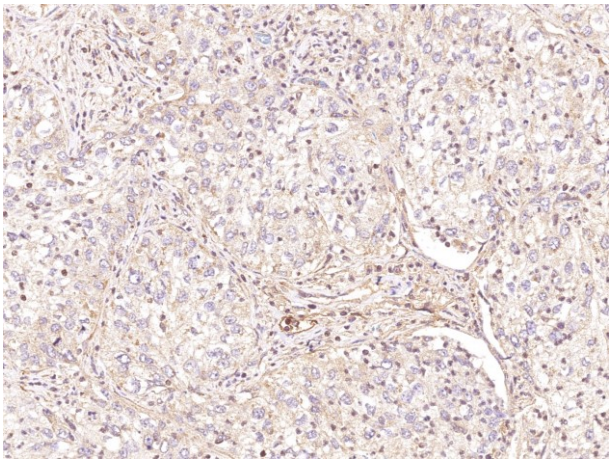


Fig. 5b
Insufficient Podop staining reaction in NSCLC using same protocol as in Fig. 4b. A diffuse background reaction is seen, and the general poor signal-to-noise ratio complicates the interpretation.

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