Supplemental Digital Content, containing 3 tables, 2 figures, and method details for the western blots. The Supplemental Digital Content was not copyedited by *Archives of Pathology & Laboratory Medicine*.

Supplemental Table 1. Recommended staining protocol for VENTANA FOLR1 Assay with OptiView DAB IHC Detection Kit on BenchMark ULTRA instruments.

Procedure Type	Recommended Protocol
Baking[a]	Optional[b]
Deparaffinization	4 mins (default), 72°C
Cell Conditioning (Antigen Unmasking)	ULTRA CC1, 64 minutes, 100°C
Pre-Primary Peroxidase Inhibitor	4 minutes, 36°C
Antibody (Primary)[a]	FOLR1-2.1 RxDx Assay Ab (32 minutes, 36°C) Or Negative Control Ab (32 minutes, 36°C)
OptiView HQ Linker	8 minutes (default), 36°C
OptiView HRP Multimer	8 minutes (default), 36°C
Counterstain[a]	Hematoxylin II, 4 minutes, 36°C
Post Counterstain[a]	Bluing, 4 minutes, 36°C

[a]Selectable by customer

[b]Baking is optional. May be performed on-board the instrument or performed offline.

Supplemental Table 2. Summary of analytical studies and associated acceptance criteria

	Definition	Agreement	Acceptance Criteria
Repeatability	The precision among repeated measurements taken under the same conditions (or within a single run of a procedure).	Intra-run	PPA, NPA & OPA ≥ 90%
		Intra-reader	APA & ANA ≥ 85%
Intermediate Precision	The precision among repeated measurements taken by varying some conditions but holding others constant.	Inter-day, Inter-instrument, Inter-antibody, Inter-detection	PPA, NPA & OPA ≥ 90%
		Inter-Reader	APA & ANA ≥ 85%
Reproducibility	The precision of repeated measurements taken under varying conditions including different laboratories, operators, and equipment.	Overall Agreement in Interlaboratory Reproducibility Study	PPA and NPA ≥ 85%

PPA, Positive Percent Agreement. NPA, Negative Percent Agreement. OPA, Overall Percent Agreement

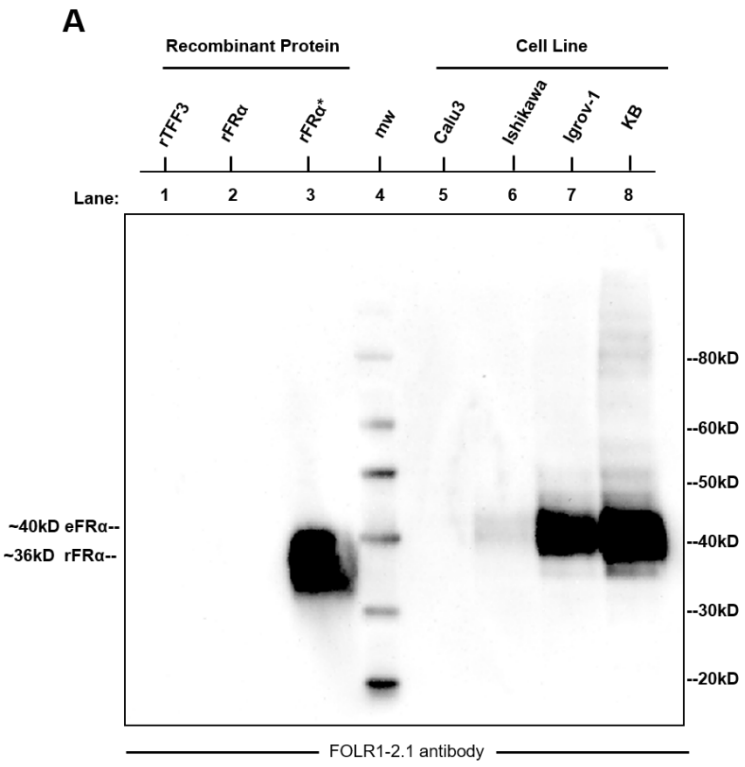
APA, Average Positive Agreement. ANA, Average Negative Agreement

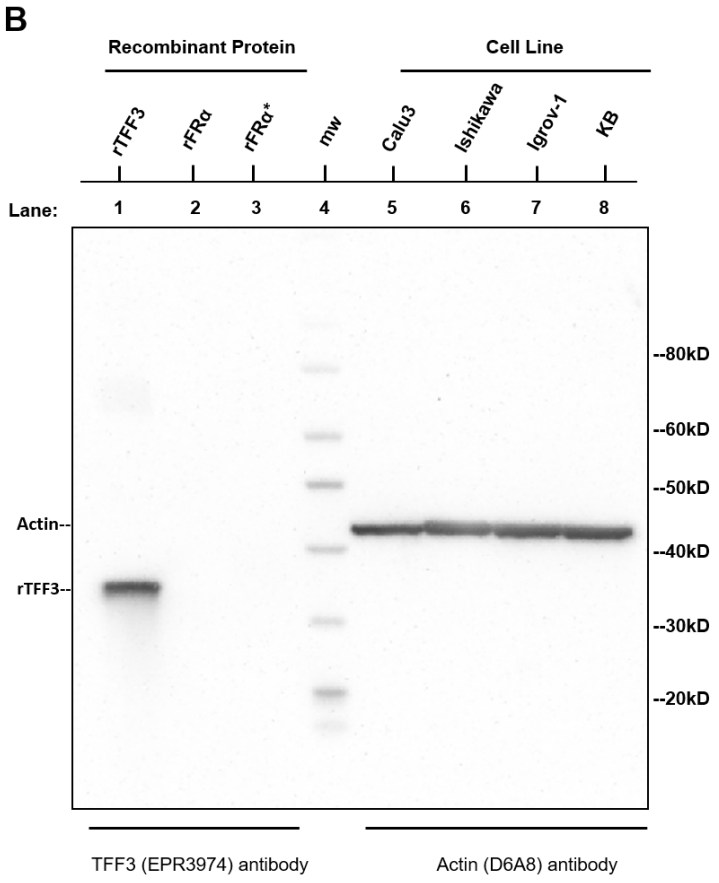
Supplemental Table 3. Cell lines used for Western Blot Analysis

Cell Line	Tissue/Disease	Stain Intensity[a]	mRNA[b]
Calu-3	Lung/Adenocarcinoma	0+	1,324
Ishikawa	Endometrial/Adenocarcinoma	1+, few cells stained	12,704
Igrov-1	Ovarian/Adenocarcinoma	2+	129,224
KB	Epithelial/Carcinoma	3+	207,626

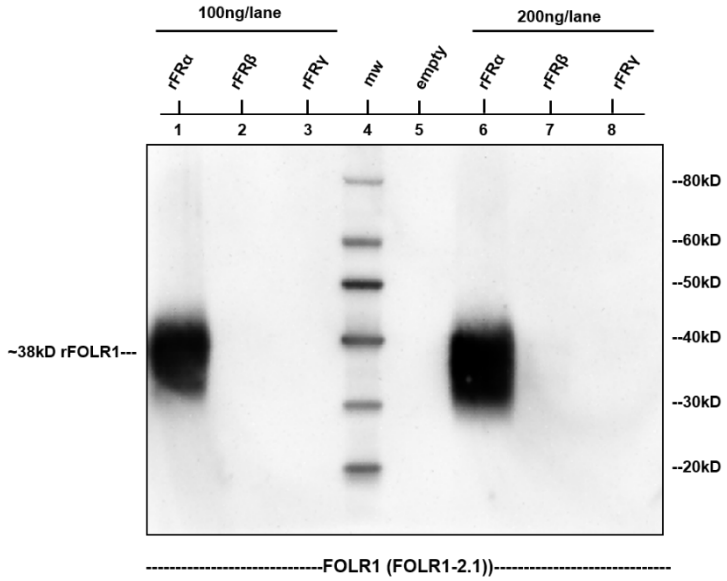
[a]Cell line immunohistochemical staining intensity was determined using the VENTANA FOLR1 Assay

[b]mRNA expression data was obtained from Genevestigator


A
Recombinant Protein
Cell Line
rTFF3
rFRα
rFRα*
mw
Calu3
Ishikawa
Igrov-1
KB
Lane:
1
2
3
4
5
6
7
8
~40kD eFRα--
~36kD rFRα--
--80kD
--60kD
--50kD
--40kD
--30kD
--20kD
FOLR1-2.1 antibody


B
Recombinant Protein
Cell Line
rTFF3
rFRα
rFRα*
mw
Calu3
Ishikawa
Igrov-1
KB
Lane:
1
2
3
4
5
6
7
8
Actin--
rTFF3--
--80kD
--60kD
--50kD
--40kD
--30kD
--20kD
TFF3 (EPR3974) antibody
Actin (D6A8) antibody

Supplemental Figure 1. Immunological Characterization by Western Blot for the anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody. Western blot analysis was utilized to assess the sensitivity and specificity of the anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody. **(A)** In lane 1, an unrelated recombinant protein TFF3 (rTFF3) served as a negative control. The anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody detects only the glycosylated form of folate receptor-α (FRα). To demonstrate that the antibody detects only the N-glycosylated form of FRα protein, both glycosylated and non-glycosylated recombinant FRα proteins encoded by amino acid 25-233, and 25-234, respectively, were obtained from Abcam. The glycosylated protein was expressed in mammalian HEK293 cells, and the non–glycosylated protein was expressed in an E.coli bacterial expression system. Non-glycosylated recombinant FRα (rFRα) was loaded into lane 2 and no band was detected using the anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody. Glycosylated recombinant FRα (rFRα*) was loaded into lane 3 and the anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody detects a single band of ~36kD, the expected molecular weight of this recombinant protein (see Supplemental Material and Methods). The anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody was tested against endogenous (eFRα) protein expressed in lysates generated from cell lines selected to provide a range of FRα protein expression based on both the VENTANA FOLR1 Assay and mRNA expression levels (Supplemental Table 3). The anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody reacted with a ~40kD band in cell lysates prepared from the cell lines that express FRα (lanes 6-8). No band of this size was detected in the negative control cell line that does not express FRα (lane 5). **(B)** To ensure that equivalent amounts of each cell lysate were loaded onto the membrane, the membrane was striped to remove bound primary and second antibody, then washed and blocked. Lanes 1-3 were re-probed with Anti-TFF3 (EPR3974) rabbit monoclonal antibody to demonstrate the presence of the recombinant negative control rTFF3 protein on the membrane. Lanes 5-8 were re-probed with anti-Actin (D6A8) rabbit monoclonal antibody.



Supplemental Figure 2. Specificity of the anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody. To demonstrate that the anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody is specific to FRα, equivalent amount of recombinant FRα (rFRα), folate receptor-beta (rFRβ) and folate receptor-gamma (rFRγ) were loaded onto a gel at 100ng and 200ng quantities. The recombinant proteins used in this experiment were all prepared in mammalian cells, with FRα and FRβ expressed in Chinese Hamster cells, and FRγ expressed in NSO-derived mouse myeloma cells. The anti-FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody detected a significant signal in lanes 1 and 6 loaded with rFRα at 100ng and 200ng, respectively. No bands were detected in any of the lanes loaded with rFRβ or rFRγ, demonstrating a lack of reactivity with these proteins. This result suggests that the anti-

FOLR1 (FOLR1-2.1) mouse monoclonal primary antibody does not cross-react with additional FR family members in the Western Blot Assay.

Supplemental Materials and Methods

The amount of recombinant proteins loaded into a gel was based on the product data sheet or certificate of analysis to load equivalent concentrations of each purified protein. Cell equivalents were used for cell lysates with volumes adjusted based on the loading control Actin to ensure that equivalent amounts of lysate were present for comparison. Recombinant FRα (rFRα) was obtained from Abcam (ab202257, amino acids 25-234) and expressed in an E.coli bacterial system and was not glycosylated in the bacterial expression system. Glycosylated recombinant FRα (rFRα*) was obtained from Abcam (ab167698, amino acids 25-233) and expressed in mammalian HEK293 cells. Recombinant TFF3 was obtained from Abnova (H00007033-P01).

Antigens were denatured in LDS sample buffer containing 2-mercaptoethanol, and diluted with H2O so that all final volumes were equivalent. Samples were then heated to 60°C for 5 minutes and allowed to cool before loading. The gel was electrophoresed in the XCell SureLock Mini cell unit for 90 minutes at 180-200 volts. Upon completion of electrophoresis, the gels were transferred to 0.45 um PVDF membranes for 7 minutes at 25 volts in in a semi-dry transfer apparatus. After transfer, the membranes were blocked for 30 minutes, washed with manufacturer's suggested buffers and then probed with each specific primary antibody diluted to a final volume of 10 ml for the overlay incubation of 60 min at room temperature or overnight at 4°C on a shaker table. The membranes were then washed and probed with the corresponding species secondary antibody conjugated to alkaline phosphatase provided in the Western Breeze kit (Thermo Fisher) and incubated for 30 minutes at room temperature on a shaker. The membranes were washed, and antibody binding was detected with a chemiluminescent substrate for alkaline phosphatase, according to the manufacturer's protocol (Thermo Fisher Western Breeze Protocol).The membrane was imaged using the FluorChem HD2 Imager (Cell Biosciences).

After the initial images were collected, the membrane shown in Supplemental Figure 1A was stripped of the primary and secondary antibodies using Restore Buffer stripping buffer (Thermo Scientific), followed by a series of wash steps. The membrane was then blocked for 30 minutes. Portions of this membrane were then probed with different primary antibodies using the methods described above, and this series of Western Blot images were used to generate Supplemental Figure 1B.

The apparent molecular weights for all protein bands were determined based on comparisons of bands to a series of molecular weight standards, MagicMark XP which can be observed by chemiluminescence (Thermo Fisher).