CANCER RESEARCH ANTIBODIES HER2 AND PR: A BENCHMARKING STUDY

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HER2 (Human Epidermal Growth Factor Receptor 2) and PR (Progesterone Receptor) are widely used cancer markers, especially in breast cancer diagnostics. This benchmarking study compared Atlas Antibodies' TripleA Polyclonals™ and PrecisA Monoclonals™ with popular commercial antibodies for HER2 and PR. The results show that the Atlas Antibodies' antibodies are comparable to or superior to the commercial antibodies in terms of specificity and efficacy in western blot, immunofluorescence, and immunohistochemistry applications for protein expression studies on human cancer tissue and cell lines.

INTRODUCTION

Cancer is a very complex, dynamic, and heterogenous disease. The hallmarks of cancer include sustained proliferative signaling, evading growth suppressors, resistance to cell death, replicative immortality, increased angiogenesis as well as invasion and metastasis. Underlying all these processes is the dysregulation of numerous proteins and protein functions in cells.

Antibodies can offer a reliable tool for specific detection of single proteins in liquid-based assays, such as ELISA, cell lysates (Western blot, WB), as well as in-situ protein detection in tissues (Immunohistochemistry, IHC) and cells (Immunocytochemistry, ICC).

Specific and sensitive primary antibodies are crucial for accurate assessment of protein expression across tissues and cells. Thus, thorough validation of antibodies in various applications is necessary to ensure accurate and reproducible results. Several approaches can be used to rigorously validate antibodies, such as genetic modification of protein expression, correlation of protein expression levels detected by antibody staining to mRNA expression levels, and comparison between independently developed antibodies targeting the same protein.

AIM

The aim of the present study was to compare Atlas Antibodies' TripleA Polyclonals™ and PrecisA Monoclonals™ against two commonly used pathology targets HER2 (Receptor tyrosine-protein kinase erbB-2, ERBB2) and PR (Progesteron receptor, PGR) to the highly-cited commercial antibodies for research use/laboratory developed assays, including Anti-HER2 Rab mAb clone SP3 (*available from ThermoFisher Scientific, Cell Marque and Zytomed; 102 published scientific references on CiteAB*) and Anti-PR Rab mAb clone SP2 (*available from ThermoFisher Scientific and Diagnostic Biosystems; 147 published scientific references on CiteAB*).

See summary "at a glance" on page 2.

ATLAS ANTIBODIES

HER2 AND PR BENCHMARKING STUDY AT A GLANCE

OBJECTIVE	Compare the performance of Atlas Antibodies' TripleA Polyclonals™ and PrecisA Monoclonals™ with widely accepted commercial antibodies.
TARGETS	**HER2:** Receptor tyrosine-protein kinase (also known as ERBB2) **PR:** Progesterone receptor (also known as PGR)
SCOPE	Assess the specificity and efficacy of these antibodies in the context of cancer research.
ATLAS ANTIBODIES' PRODUCTS	**Anti-ERBB2 (HER2):** - Triple A Polyclonals HPA001383 and HPA001338 - PrecisA Monoclonal AMAb90627 **Anti-PGR (PR):** - Triple A Polyclonals HPA008428 and HPA004751 - PrecisA Monoclonal AMAb91529
COMPARISON GROUP	**Anti-HER2 Rab mAb clone SP3** (available from ThermoFisher Scientific, Cell Marque, and Zytomed; 102 published scientific references on CiteAB). **Anti-PR Rab mAb clone SP2** (available from ThermoFisher Scientific and Diagnostic Biosystems; 147 published scientific references on CiteAB).
METHODOLOGY	All antibodies underwent rigorous testing in three key applications ensuring a thorough evaluation across diverse experimental settings: - Western blot (WB). - Fluorescence immunocytochemistry (ICC-IF) using cell cultures. - Chromogenic immunocytochemistry (ICC) using dynamic range cell line controls. - Immunohistochemistry (IHC) utilizing a variety of normal and cancerous human tissues with annotated HER2 and PR expression.
RESULTS	**Anti-ERBB2, Triple A Polyclonal Antibody (HPA001383)**: - Superior specificity compared to commonly used clone SP3 (ThermoFisher Scientific, Cat # MA5-14509). - Demonstrates excellence in WB, ICC-IF, and IHC applications. **Anti-PGR, PrecisA Monoclonal Antibody (AMAb91529)**: - Superior sensitivity compared to clone SP2 (ThermoFisher Scientific, Cat # MA5-14505) in IHC application. - Comparable results in WB and ICC-IF.
SIGNIFICANCE	In a nutshell, the anti-ERBB2 and anti-PGR Triple A Polyclonal and PrecisA Monoclonal antibodies from Atlas Antibodies are comparable to or superior to their highly cited and widely recognized antibody counterparts (clone SP3 and clone SP2, targeting HER2 and PR, respectively) in specific applications.

MATERIALS AND METHODS

All antibodies were tested in western blot (WB), fluorescence immunocytochemistry (ICC-IF), chromogenic immunocytochemistry (ICC) and immunohistochemistry (IHC) applications using relevant cell lysates (WB) and cell cultures (ICC-IF), dynamic range cell line controls (ICC) and finally a range of normal and cancerous human tissues (IHC) with annotated HER2 and PR expression. Details on antibodies used, applications tested, and working dilutions/concentrations are displayed in Table 1.

Western blot

WB analyses were performed according to standard protocols on mammalian cell lysates, including SK-BR-3 (Cat # ACC-736, DSMZ Leibniz Institute, Braunschweig, Germany), RT-4 (Cat # ACC-412, DSMZ Leibniz Institute, Braunschweig, Germany), PC-3 (Cat # ACC-465, DSMZ Leibniz Institute, Braunschweig, Germany), MCF-7 (Cat # 86012803, ECCAC, Salisbury, UK) for HER2 antibodies and T-47D (Cat # 85102201, ECACC, Salisbury, UK) for PR antibodies.

Briefly, 20 µg of lysate was separated on precast 4–20 % Criterion TGX SDS-PAGE gradient gel (Bio-Rad Laboratories, Hercules, CA) under reducing conditions, followed by blotting to PVDF membranes (Trans-Blot® Turbo™ Midi PVDF Transfer Packs, Bio-Rad Laboratories, Hercules, CA), according to the instructions of the manufacturer. Membranes were blocked for 30 min at room temperature (RT) in blocking buffer (5 % dry milk, 0.5 % Tween 20, 1× TBS) prior to addition of a corresponding antibody, which was diluted to a final concentration of 1 µg/mL in blocking buffer.

Following incubation for 1 h with primary antibody, the membranes were washed 4 times utilizing a SNAP i.d. 2.0 Protein Detection System-Midi (Merck KGaA, Darmstadt, Germany) with 1× TBS and 0.1 % Tween 20. Horseradish peroxidase (HRP)-conjugated secondary antibody (swine anti-rabbit antibody #P0399 or goat anti-mouse antibody #P0447, Dako) was diluted 1:3,000 in blocking buffer and added to the membranes for 10 min incubation, followed by a final round of washing.

Detection was carried out using a chemiluminescence HRP substrate (Immobilon, EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions.

Immunofluorescence

ICC-IF analyses were performed according to standard protocols in mammalian cells, including SK-BR-3 and T-47D. Briefly, a glass bottomed plate was coated with fibronectin for 1h at room temperature. Cells were then seeded and incubated at 37°C for 24 hours in humified air with 5% CO2 in growth medium. Growth medium was removed, and cells were washed in Dulbecco's Phosphate Buffered Saline (DPBS). Methanol-free formaldehyde (4%) in DPBS was added for fixation for 15 min. Cells were then permeabilized with 0.1% Triton X-100 in DPBS for 10 min.

Cells were washed with DPBS and blocking buffer (4% FBS in DPBS) was added for 1h before the addition of primary antibodies. The plate with cells and primary antibodies diluted in blocking buffer was incubated overnight at 4°C.

Cells were then washed for 3x10 min with DPBS and incubated with secondary antibodies in blocking buffer for 1.5h at RT. DAPI nuclear counterstain (Invitrogen) was applied at 1 ug/mL in DPBS before a final wash with DPBS for 3x10 min and mounting in DPBS with 0.02 % (w/v) NaN3.

Primary antibodies against HER2 and PR were tested at a working concentration of 0.5-10 µg/mL (see Table 1). In addition, mouse anti-alpha Tubulin monoclonal antibody Ab7291 (Abcam) diluted 1:500 was used as a cytoskeletal marker for Triple A Polyclonals and rabbit monoclonal antibodies, clones SP3 and SP2. Rabbit anti-alpha Tubulin monoclonal antibody ab52866 (Abcam) diluted 1:1000 was used as a cytoskeletal marker for PrecisA Monoclonals.

Secondary antibodies used for immunofluorescence detection include:

- Alexa® Fluor 488 goat anti-rabbit IgG (ThermoFisher Scientific, no: A11034) diluted 1:400,
- Alexa® Fluor 594 goat anti-mouse IgG (ThermoFisher Scientific, no: A11032) diluted 1:400,
- Alexa® Fluor Plus 488 goat anti-mouse IgG (ThermoFisher Scientific, no: A32723) diluted 1:800, and
- Alexa® Fluor 594 goat anti-rabbit IgG (ThermoFisher Scientific, no: A11037) diluted 1:400.

Cells were examined and images were taken using a confocal Leica TCS SP8X microscope.

Immunohistochemistry in tissues and cells

IHC analyses were performed according to standard protocols on human tissue microarrays (TMA, containing 18 normal and 7 cancer tissues, constructed by Atlas Antibodies) and breast cancer sections with defined HER2 and PR status (all tissues were purchased from commercial biobanks, including Asterand®, BioIVT, West Sussex, UK; Tissue Solutions Ltd, Scotland, UK; and Indivumed GmbH, Hamburg, Germany).

In addition, dynamic range cell line microarrays (CMAs) were used as protein expression level controls (HER2 Analyte Control DR, Cat # HCL028 and PR Analyte Control DR, Cat # HCL034, both from Histocyte Laboratories, Newcastle upon Tyne, UK).

Four µm sections were cut from the TMA and breast cancer formalin fixed paraffin embedded (FFPE) tissue blocks as well as the CMAs. CMA sections were mounted on the same slides as the breast cancer sections. Prior to immunostaining, slides were baked at 50 °C overnight and deparaffinized in xylene and graded ethanol.

Antigen retrieval was performed using citrate buffer at pH 6 (ThermoFisher Scientific, Waltham, MA, USA) in a decloaking chamber (NxGen, Biocare Medical). Sections were stained in an Autostainer 480S (ThermoFisher Scientific, Waltham, MA, USA) using primary antibodies (incubated for 30 min at RT, see Table 1 for dilutions) and a standard commercial detection kit (UltraVision LP HRP polymer®, Primary Antibody Enhancer, Ultra V Block, ThermoFisher Scientific, Waltham, MA, USA and Bright DAB substrate system®, Immunologic, Arnhem, The Netherlands).

Slides were counterstained with hematoxylin and mounted using Pertex (both from Histolab, Gothenburg, Sweden). Images were taken using a Zeiss Axio Imager.Z2 microscope equipped with an automated scanning system (VSlide, Metasystems, Altlussheim, Germany).

Table 1.

Summary of antibodies, working dilutions/concentrations and applications tested in the benchmarking study.

	Supplier	Product ID	Clone ID	Clonality	Conc, mg/ml	Applications tested			References
						WB Conc	ICC-IF Conc	IHC/ICC Dil	
HER-2	ThermoFisher	MA5-14509	SP3	Rb mAb	0.1	1 µg/ml	2 µg/ml	1:250	102
	Atlas Antibodies	HPA001383	N/A	Rb pAb	0.1	1 µg/ml	1 µg/ml	1:500	8
	Atlas Antibodies	HPA001338	N/A	Rb pAb	0.05	1 µg/ml	1 µg/ml	1:500	
	Atlas Antibodies	AMAb90627	CL0628	Mo mAb	1	1 µg/ml	10 µg/ml	1:100	2
PR	ThermoFisher	MA5-14505	SP2	Rb mAb	0.1	1 µg/ml	2 µg/ml	1:400	147
	Atlas Antibodies	HPA004751	N/A	Rb pAb	0.1	1 µg/ml	1 µg/ml	1:250	
	Atlas Antibodies	HPA008428	N/A	Rb pAb	0.05	1 µg/ml	0,5 µg/ml	1:20	
	Atlas Antibodies	AMAb91529	CL8721	Mo mAb	1	1 µg/ml	5 µg/ml	1:4000	

RESULTS

Anti-ERBB2 (HER2) antibodies validation

All four antibodies showed comparable results in WB application, displaying a band of expected size when tested in a high-expressing cell line SK-BR-3 (RNA levels= 2343 TPM) and absence of signal in a low-expressing cell line MCF-7 (RNA levels = 45 TPM) (Figure 1A-D). However, when tested on two lower-expressing cell lines (RT-4 vs PC-3, see relative mRNA expression levels in Figure 1G), the clone SP3 displayed several additional non-specific bands (Figure 1E), while HPA001383 showed only a single expected band in a higher-expressing RT-4 cell line (Figure 1F).

Antibodies were then tested in ICC-IF application using the SK-BR-3 cell line. Clone SP3 (Figure 2A) and Triple A Polyclonals HPA001383 (Figure 2B) and HPA001338 (Figure 2C) all displayed membranous immunoreactivity as expected. Remarkably, the signal intensity was higher in HPA001383 and HPA001338 tested at 1 µg/mL compared with the SP3 clone tested at 2 µg/mL (Figure 2 A-C). As the PrecisA Monoclonal AMAb90627 is not approved for use in ICC-IF, only negative results were observed using this antibody as expected (Figure 2D).

ICC validation using dynamic range cell line controls with HER2 expression ranging from negative to 1+, 2+, and 3+, showed comparable results for all four antibodies. Absence of signal was observed in the negative cell line (Figure 3A, E, I, M), moderate membranous positivity in a subset of cells was detected in Her2 1+ cell line (Figure 3B, F, J, N), moderate membranous immunoreactivity in the majority of cells was seen in the HER2 2+ cell line (Figure 3C, G, K, O), and finally strong membranous staining in all cells was observed in HER2 3+ cell line (Figure 3D, H, L, P).

When tested for IHC application in a number of control human tissues, antibodies displayed differential results. All four antibodies displayed expected strong membranous immunoreactivity in HER2 positive breast cancer sample (Figure 4 A, F, K, P) and absence of positivity in HER2 negative breast sample (Figure 4B, G, L, Q). Weak membranous immunoreactivity was detected with all antibodies in distal tubules in kidney as expected (Figure 4C, H, M, R).

However, clone SP3 displayed additional non-specific positivity in negative control tissues, such as testis, where nucleolar immunoreactivity was observed (Figure 4D, see also inset at 2x higher magnification), and cytoplasmic staining in skeletal muscle (Figure 4E). All other antibodies, including HPA001383, HPA001338 and AMAb90627 displayed no staining in negative control tissues as expected (Figure 4 I, J, N, O, S, T).

Anti-PGR (PR) antibodies validation

In WB application, all four antibodies showed comparable results and displayed a double band of expected size (corresponding to PR-A and PR-B isoforms of the protein), when tested in a high-expressing cell line T-47D (Figure 5 A-D).

Also, in ICC-IF application, all four antibodies displayed similar results, namely nuclear positivity in T-47D cell line (Figure 6 A-D).

When tested in IHC application using dynamic range cell line controls with PR expression ranging from negative to low/moderate, moderate/high to high, expected results were observed using clone SP2 (Figure 7 A-D), as well as HPA008428 (Figure 7 I-L) and AMAb91529 (Figure 7 M-P).

HPA004751 (Figure 7 E-H) displayed some background positivity in the presumably negative cell line (Figure 7E, see also inset at 2x higher magnification) when tested at 1:250 dilution, suggesting that this antibody could require further titration/optimization.

When IHC was performed on the control FFPE human tissues, all antibodies could successfully detect PGR positivity in the PR-positive breast cancer sample (Figure 8A, E, I, M) and were negative in the PR-negative breast cancer sample (Figure 8B, F, J, N).

Furthermore, normal tissues such as fallopian tube used as positive control and cerebral cortex used as negative control displayed either strong immunoreactivity (Figure 8C, G, K, O) or absence of positivity (Figure 8D, H, L, P) as expected, when probed with all four antibodies.

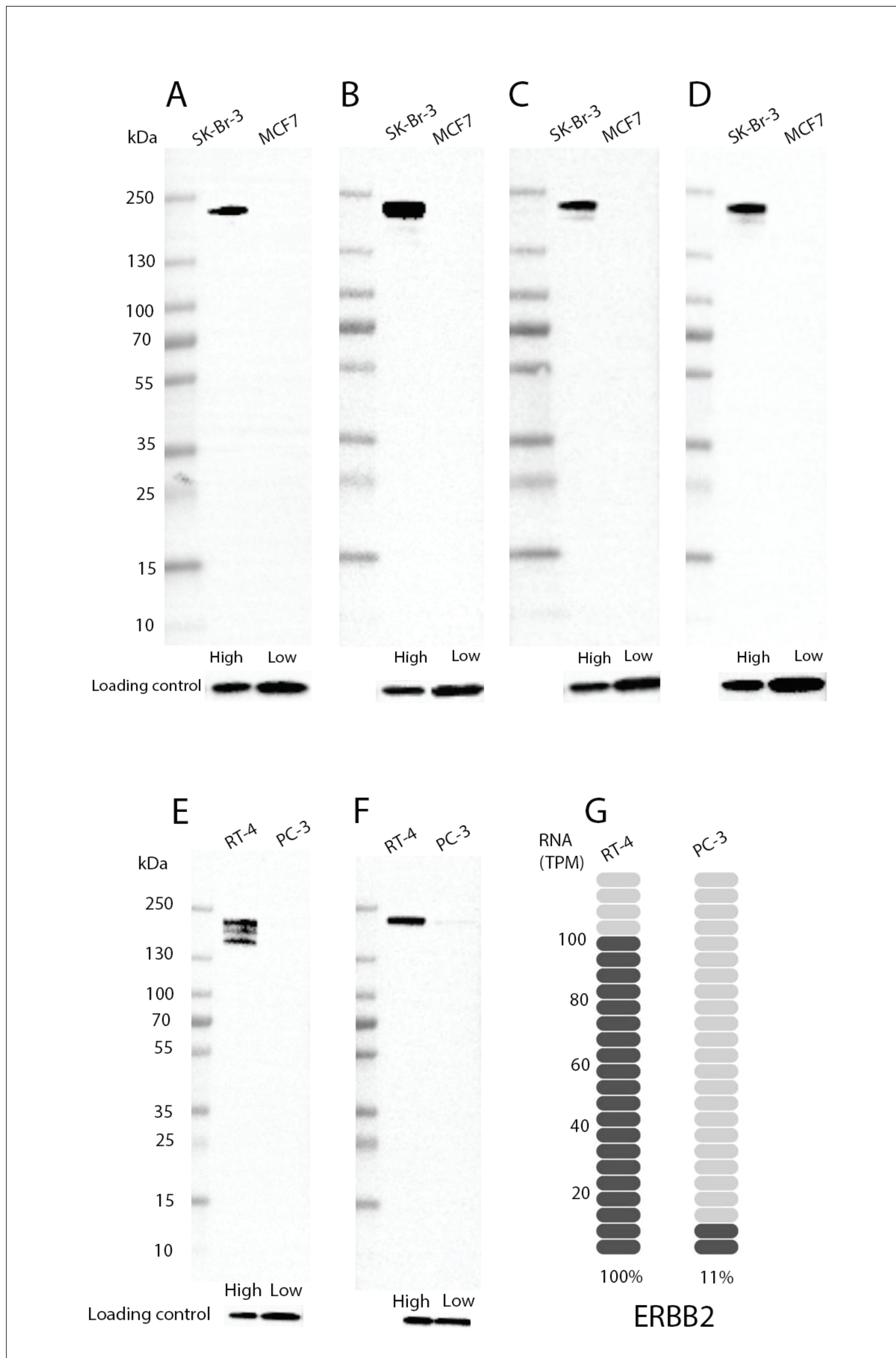

Figure 1. HER2 in WB

Anti-ERBB2 (HER2) antibody validation in WB application using SK-BR-3 (high-expressing) and MCF-7 (low expressing) cell lysates (**A-D**) and MA5-14509 Clone SP3 (**A**), HPA001383 (**B**), HPA001338 (**C**), and AMAb90627 (**D**) antibodies. Anti-COX4L1 antibody AMAB91171 was used as loading control.

Additional validation of MA5-14509 Clone SP3 (**E**) and HPA001383 (**F**) in RT-4 (high-expressing) and PC-3 (low expressing) cell lysates. RNA expression levels for RT-4 and PC-3 cell lines are shown in **G**. Anti-COX4L1 antibody AMAB91171 was used as loading control.

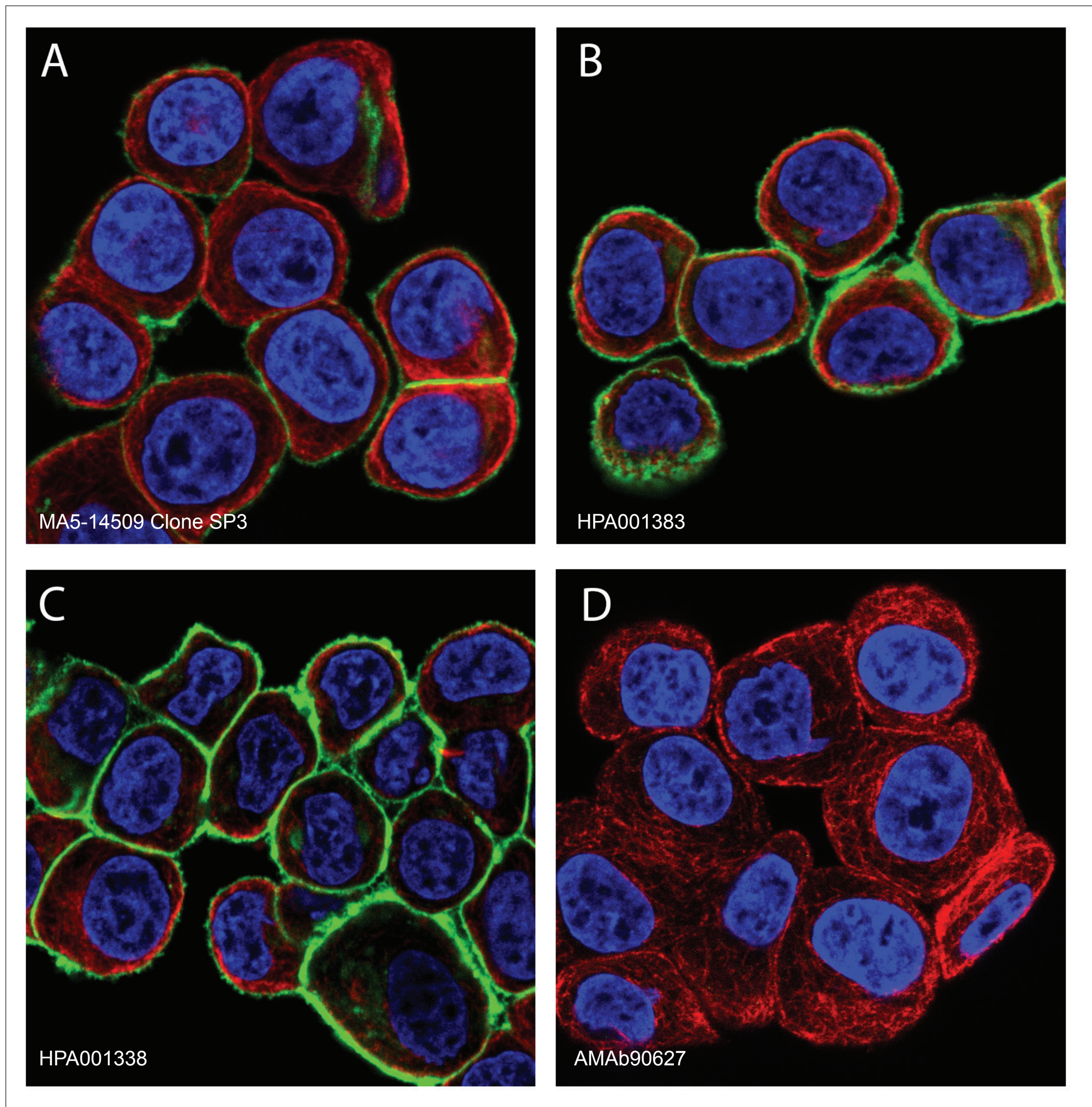

Figure 2. HER2 in ICC-IF

Anti-ERBB2 (HER2) antibody validation in ICC-IF application using SK-BR-3 cell line and MA5-14509 Clone SP3 (**A**), HPA001383 (**B**), HPA001338 (**C**), and AMAb90627 (**D**) antibodies.

Green immunofluorescence signal shows expected localization in plasma membrane for MA5-14509 Clone SP3 (**A**), HPA001383 (**B**), HPA001338 (**C**), and absence of signal for AMAb90627 (**D**). Microtubules and nuclear counterstain are displayed in red and blue respectively.

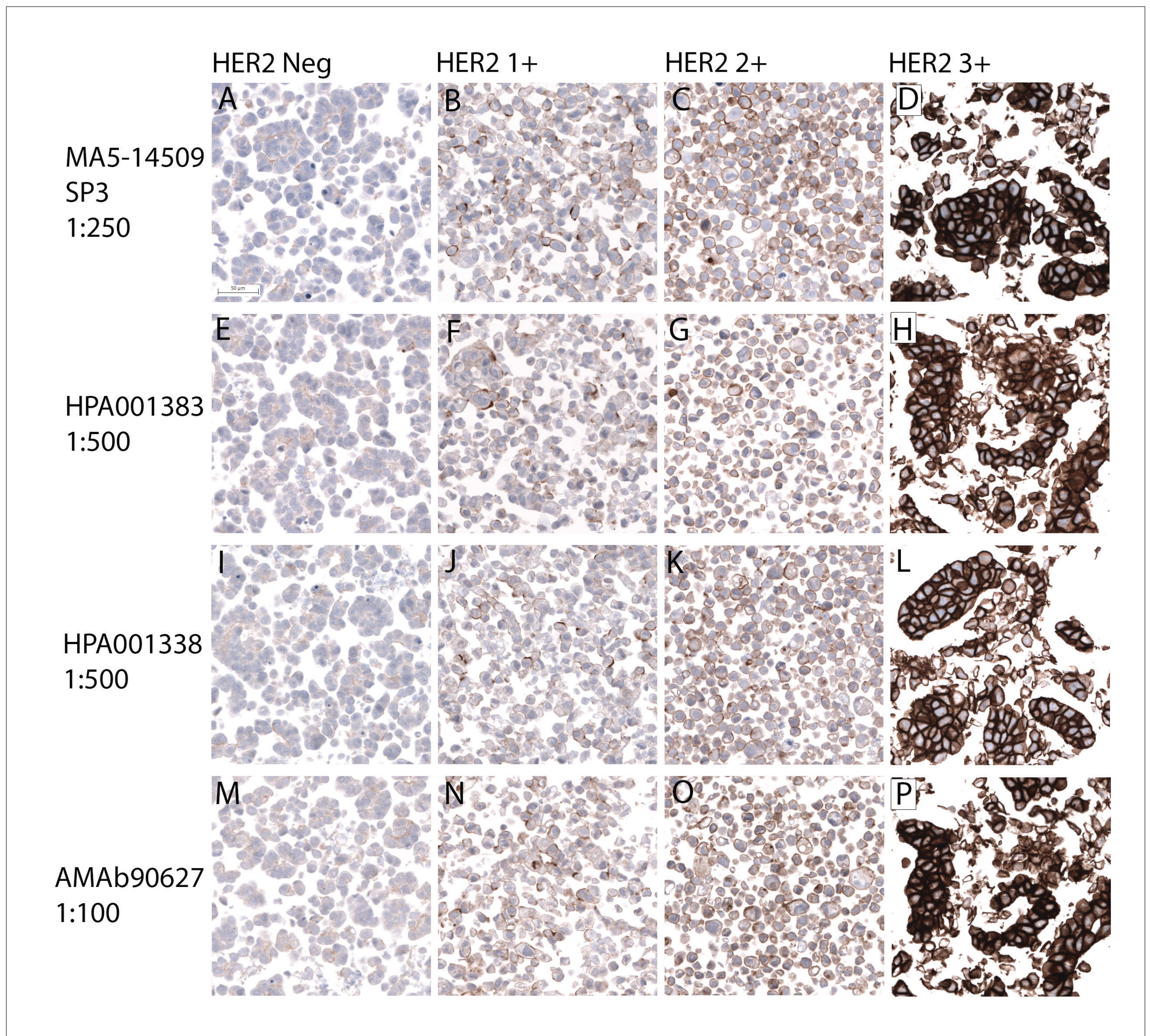

Figure 3. HER2 in cell line controls

Anti-ERBB2 (HER2) antibody validation in IHC application using dynamic range cell line controls with annotated protein expression and MA5-14509 Clone SP3 (**A-D**), HPA001383 (**E-H**), HPA001338 (**I-L**), and AMAb90627 (**M-P**) antibodies. Scale bar (on A) indicates 50 µm.

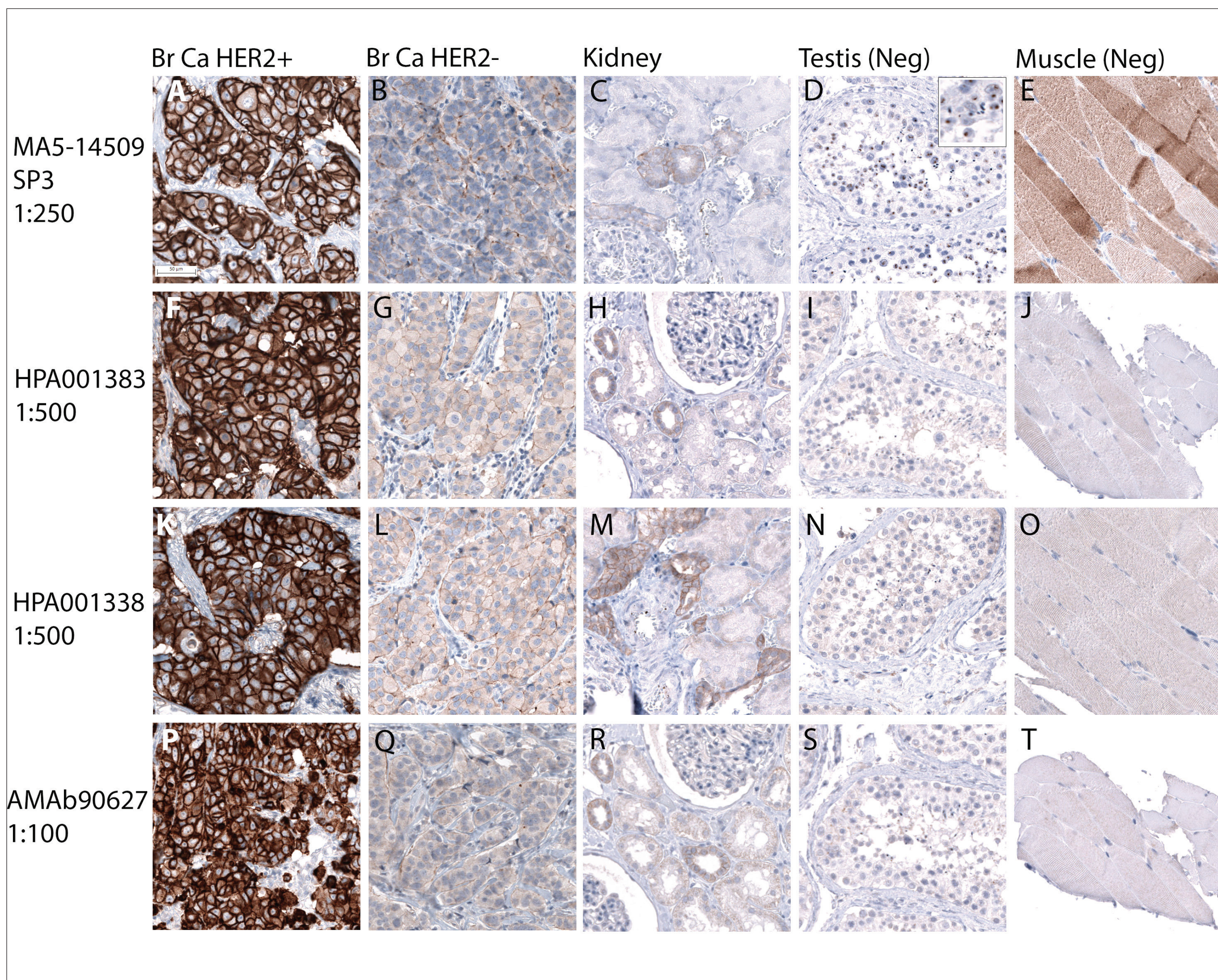

Figure 4. HER2 in tissues

Anti-ERBB2 (HER2) antibody validation in IHC application employing human FFPE positive and negative control tissues, using MA5-14509 Clone SP3 (**A-E**), HPA001383 (**F-J**), HPA001338 (**K-O**), and AMAb90627 (**P-T**) antibodies. Scale bar (on A) indicates 50 µm.

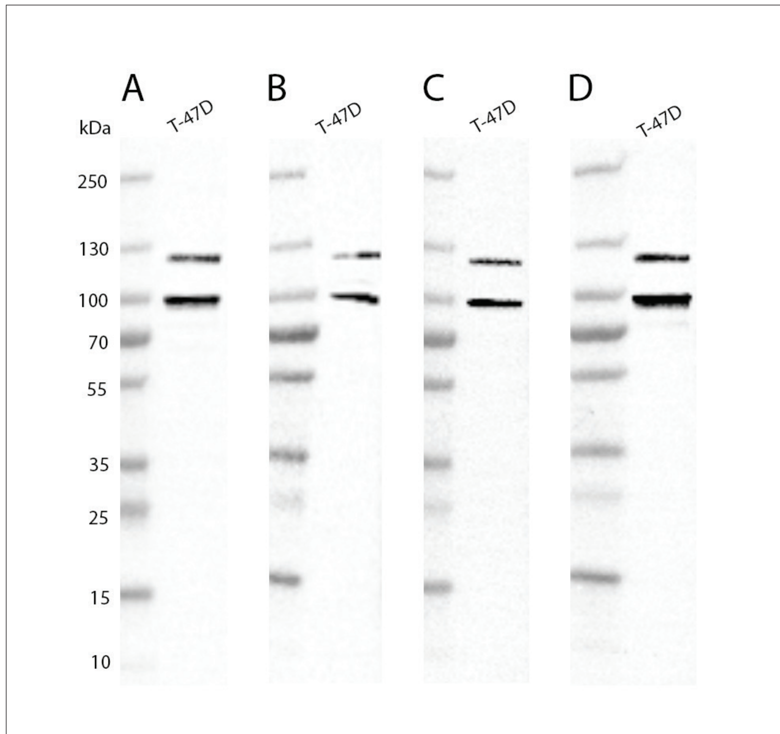

Figure 5. PGR in WB

Anti-PGR (PR) antibody validation in WB application using a high-expressing T-47D cell line and MA5-14505 Clone SP2 (**A**), HPA004751 (**B**), HPA008428 (**C**), and AMAb91529 (**D**).

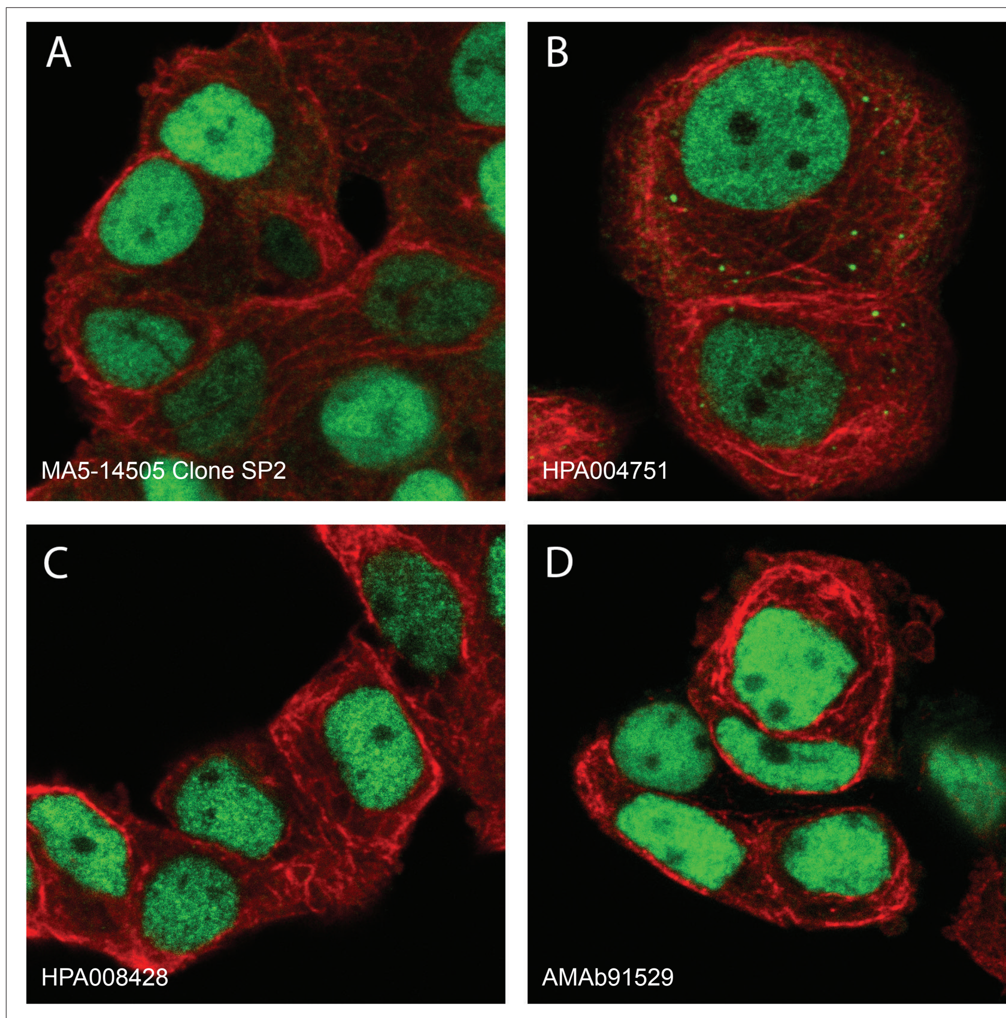

Figure 6. PGR in ICC-IF

Anti-PGR (PR) antibody validation in ICC-IF application using T-47D cell line and MA5-14505 Clone SP2 (**A**), HPA004751 (**B**), HPA008428 (**C**), and AMAb91529 (**D**).

Green fluorescence signal shows expected localization to cell nucleus. Microtubules are displayed in red.

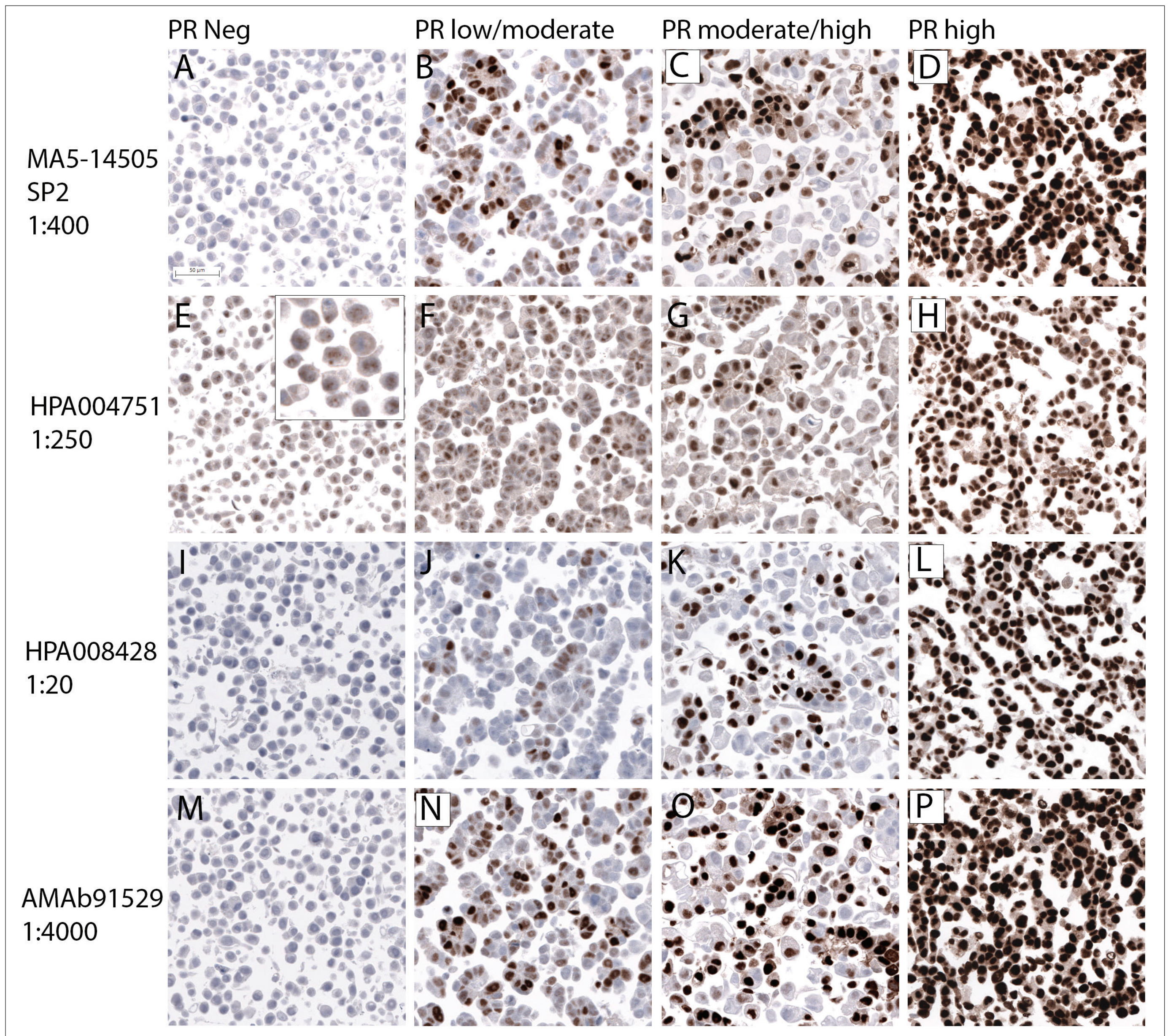

Figure 7. PGR in cell line controls

Anti-PGR (PR) antibody validation in IHC application using dynamic range cell line controls with annotated protein expression and MA5-14505 Clone SP2 (**A-D**), HPA004751 (**E-H**), HPA008428 (**I-L**), and AMAb91529 (**M-P**). Scale bar (on A) indicates 50 μm.

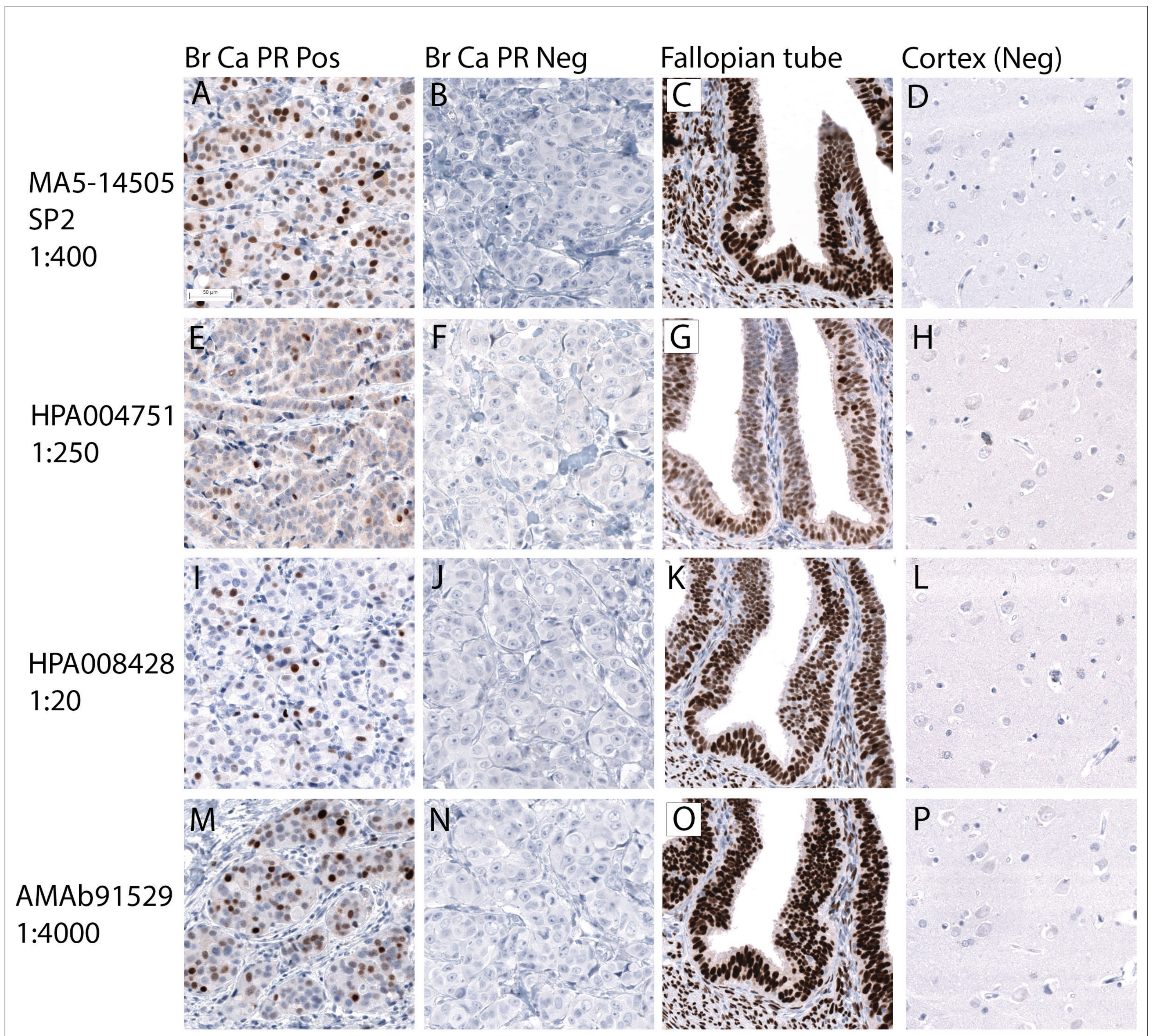

Figure 8. PGR in tissues

Anti-PGR (PR) antibody validation in IHC application using human FFPE positive and negative control tissues and MA5-14505 Clone SP2 (**A-D**), HPA004751 (**E-H**), HPA008428 (**I-L**) and AMAb91529 (**M-P**). Scale bar (on A) indicates 50 µm.

DISCUSSION AND CONCLUSION

In surgical pathology, detection of protein expression can be used for diagnosis of the particular tumor types or differential diagnosis between histologically similar tumors.

Protein expression levels are an essential component which determines how patients are treated, called companion diagnostics, (e.g. Herceptin test in breast cancer). Consequently, accurate and reproducible antibody-based assays are paramount to avoid potential harm of false-positive or negative results due to inadequate antibody validation.

For these reasons, extensive validation of antibodies across applications using a range of control lysates, tissues and cell lines ensures accurate and reproducible results.

The results of the present benchmarking evaluation indicate that the Triple A Polyclonal antibody HPA001383 from Atlas Antibodies, targeting HER2/ERBB2, shows superior specificity compared to the commonly used clone SP3 (ThermoFisher Scientific, Cat #MA5-14509) in WB and IHC applications.

The PrecisA Monoclonal antibody AMAb91529 from Atlas Antibodies, targeting PR/PGR, displays superior sensitivity to that of clone SP2 (ThermoFisher Scientific, Cat #MA5-14505) in IHC, and comparable results in both WB and ICC-IF applications.

Taken together, the results of this study demonstrate that Atlas Antibodies' products can be successfully used in protein expression studies and display comparable or superior results compared to highly cited and commonly used antibodies on the market.

KEY TAKEAWAYS

The aim of the study was to compare Atlas Antibodies' Triple A Polyclonals™ and PrecisA Monoclonals™ with commonly used commercial antibodies for HER2 and PR in various applications such as western blot, immunofluorescence, and immunohistochemistry.

The results of the study showed that the Triple A Polyclonal antibody HPA001383 targeting HER2 demonstrated superior specificity compared to the commonly used clone SP3 in western blot and immunohistochemistry applications, and similar results in immunofluorescence.

The PrecisA Monoclonal antibody AMAb91529 targeting PR displayed superior sensitivity compared to the clone SP2 in immunohistochemistry application, and comparable results in western blot and immunofluorescence.

All antibodies were validated using a range of control cell lines, tissues, and microarrays to ensure accurate and reproducible results.

The study concludes that Atlas Antibodies' products can be successfully used in protein expression studies with results comparable to or superior to highly cited and commonly used antibodies on the market.

ATLAS ANTIBODIES ADVANCED POLYCLONALS.
Triple A Polyclonals™ are rabbit polyclonal primary antibodies developed within the Human Protein Atlas project. IHC characterization data from 44 normal and 20 cancer tissues is available on the Human Protein Atlas portal.

PRECISE. ACCURATE.TARGETED.
PrecisA Monoclonals™ are mouse monoclonal primary antibodies developed against a number of carefully selected targets. Clones are selected to recognize only unique non-overlapping epitopes and isotypes.

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