# A Clean and Sensitive Method for Detection of Human Host Primary Antibodies on Human Tissues

Rachel M Gonzalez, Li Ding, Ming Xiao, Bob Melton, Xiaoping Zhang

GBI Labs-Inc, Mukilteo, WA 98275  info@gbi-inc.com

**GBI Labs** GOLDEN BRIDGE INTERNATIONAL

**Abstract:** Studies have shown that targeting tumors with tumor specific antibody drug conjugates is more effective treatment than treatment with free drug, antibody alone, or a mixture of drug and antibody. Greater than 99% of antibodies to human proteins are made in animal species. However, the use of animal antibody can lead to an anti-globulin response and hypersensitivity reactions. Researchers are hoping to minimize this antigenic response by developing tumor specific human host primary antibodies. However screening human primary antibody on human tissue is difficult due to high background issues. Here we evaluate GBI Labs polymer based ready to use Klear Human kit with primary human anti-human cytokeratin 18/19 on 9 human colon cancer and 12 human breast cancer cases to show specificity of staining. In addition, we evaluated 20 different human tissues with no primary human antibody and found this detection system produces no background. We concluded that this kit provides the researcher with a tool to screen multiple human primary antibody clones for their sensitivity and specificity to the targeted human tumor and to assess the potential collateral damage on non targeted human tissues.

**Introduction:** In the past decade, researchers and pharmaceutical companies have improved the treatment of diseases by using monoclonal antibody therapy with or without drug conjugates. It is the antibody's ability to recruit other parts of the immune system to destroy targeted antigens, such as cancer cells, which makes this therapy promising. Preclinical testing for therapy application of human or humanized antibodies include studies such as antibody dose toxicity, hemolysis, functional loss of antibody by activation of the complement system, and an immunohistochemical cross-reactivity screen of multiple tissues. Although many improvements have been made in several areas of preclinical testing, rapid immunohisto chemical (IHC) screening of large sample sets have been slowed by background issues generated when screening human or humanized primary antibodies on human tissues. To improve the immunohistochemical screening process of human or humanized primary antibodies on small or large scale human tissue sets, we have developed a polymer based ready to use Klear Human kit. The Klear Human kit has an overnight priming step with the human/humanized primary antibody, followed by traditional polymer IHC-HRP/DAB application steps. In this study, we compared the staining pattern of two human(Hu) anti-Hu primary antibodies to mouse (Ms) and rabbit (Rb) primary antibodies for the same protein.

First, the primary Hu anti-Hu cytokeratin18/19 (anti-Hu Ck18/19), Ms anti-Hu Ck18, and Ms anti-Hu Ck19 was screened on 9 human colon cancer and12 human breast cancer cases using Klear Human for Hu or D12xx Polink 1 HRP for Ms primary antibody detection. The Hu anti-Hu Ck18/19 was similar to both the Ms anti-Hu Ck18 and Ms anti-Hu Ck19 staining on the colon cancer but some differences were noted in the breast cancer tissue arrays. We then screened the breast and colon cancer tissue arrays for the nuclear proliferation marker Ki67 using Hu anti-Hu Ki67, Ms anti-Hu Ki67, and Rb anti-Hu Ki67. The Ms and Rb anti-Hu Ki67 both produced nuclear staining when positive in these tissues. However, the Hu anti-Hu Ki67 showed cytoplasmic staining pattern in serial sections of the same tissue. Suggesting Hu anti-Hu Ki67 antibody does not recognize Ki67 protein in tissues even though this antibody was previously shown in ELISA and westerns to recognize Ki67. Finally, we screened for non specific staining on 20 different human tissues and showed minimal background generated at 1 hour. Our data suggest that the new Klear Human kit provides a means to rapidly screen multiple human or humanized primary antibody clones for their sensitivity and specificity to their proposed antigen target and to assess the potential of hitting non specific antigens or non targeted human tissues in large sample sets.

**Methods:** Tissue specimens were obtained from Dr. Yang, Beijing, China with only a description of tissue contained in paraffin block. No additional information about the specimen was made available. For the tumor tissue array, representative cores of the tumor were used to generate two tissue arrays, one of breast cancer and the second of colon cancer. Screens were done as follows. Four-µm sections were cut and mounted on coated slides and dried overnight at 37°C. For IHC staining on the Day 1, Hu primary antibodies were diluted in Klear Human Reagent 1 Human Primer (RTU), mixed gently for 30-60 seconds, and incubated overnight at 4°C. On Day 2, Slides were de-waxed in xylene and re-hydrated using graded alcohols then rinsed in tap water. Endogenous peroxidase activity was blocked with 3% H2O2 for 10 minutes, followed by several changes of water. **H**eat **I**nduced **E**pitope **R**etrieval (**HIER**) was required for all the primary antibodies used in this study and was done with 10mM Citrate buffer pH6.0 (GBI Labs, Mukilteo, WA, USA) for 15 min at 95-100°C. Slides were allowed to cool-down to 45°C and rinsed in water before proceeding to the next step. Slides were washed with PBS containing 0.5% tween20, 3 times for 2 minutes each after every step until DAB chromogen. To stop priming reaction, Quenching Buffer Reagent 2 was added into primary antibody mixture of Day1 and incubated at room temperature(RT) for 30 minutes then placed on ice until needed. Human Blocking Buffer A was applied on the tissue and incubated for 30 minutes at room temperature. Slides were washed and Human Blocking Buffer B was applied and incubated for 5 minutes at room temperature. Slides were washed and Human primary antibody mixture containing Human primer and Quenching Buffer was incubated on the tissue for 1-2 hours at RT. Slides were washed and Human HRP Polymer Reagent 5 was applied and incubated for 10-15 minutes. Slides were washed and DAB chromogen Reagent 6B was mixed with substrate buffer Reagent 6A and applied to slides for 5 minutes. Slides were washed in copious amounts of water then counterstained with hematoxylin. Polink-1 HRP for Ms (D12xx) or Rb(D13xx) for DAB Bulk Kit (GBI Labs, Mukilteo, WA USA) was used as a secondary detection system for primary antibody from Ms or Rb host species which were used as positive controls for human protein staining for every section. Primary antibody was applied for 30 minutes at RT. Slides were washed D12xx Polink1 Polymer HRP anti-Ms and/or D13xx Polink1 Polymer HRP anti-Rb was incubated for 15 minutes at RT. Slides were washed and DAB Chromogen (C09xx) was incubated on the tissue for 5 minutes. For background screening of Klear Human kit, the same protocol was followed, however no primary antibody was added. Tissues were scored using Olympus BX40 Light microscope.

**Table 1** Materials Used

| Primary Antibody | Antibody Titer | Source & Cat # | 2nd Detection Source / Cat # | Tested Tissue | Antigen Retrieval Buffer |
|---|---|---|---|---|---|
| **Host: Human anti-Hu CK18/19** | 1:50 | AbD Serotec HCA077A | Klear Human Kit GBI Labs / D103xx | Breast Cancer Colon Cancer | 10mM Citrate buffer pH6.0 GBI Labs / B05C-100B |
| **Host: Human anti-Hu Ki67** | 1:100 | AbD Serotec HCA117 | Klear Human Kit GBI Labs / D103xx | Breast Cancer Colon Cancer Tonsil | 10mM Citrate buffer pH6.0 GBI Labs / B05C-100B |
| **Host: Mouse anti-Hu CK18** | 1:300 | GBI Labs M3088 | Polink-1 HRP for Ms GBI Labs / D12xx | Breast Cancer Colon Cancer | 10mM Citrate buffer pH6.0 GBI Labs / B05C-100B |
| **Host: Mouse anti-Hu CK19** | 1:300 | GBI Labs M3079 | Polink-1 HRP for Ms GBI Labs / D12xx | Breast Cancer Colon Cancer | 10mM Citrate buffer pH6.0 GBI Labs / B05C-100B |
| **Host: Mouse anti-Hu Ki67** | 1:50 | GBI Labs M11-008A | Polink-1 HRP for Ms GBI Labs / D12xx | Breast Cancer Colon Cancer Tonsil | 10mM Citrate buffer pH6.0 GBI Labs / B05C-100B |
| **Host: Rabbit anti-Hu Ki67** | 1:100 | Thermo RM-9106-S | Polink-1 HRP for Rb GBI Labs / D13xx | Breast Cancer Colon Cancer Tonsil | 10mM Citrate buffer pH6.0 GBI Labs / B05C-100B |

## Results:

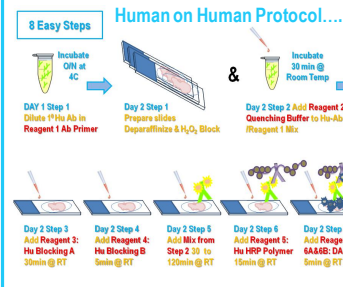**Figure 1** Cartoon of Klear Human staining protocol to screen a human primary antibody on human tissue.



Human on Human Protocol....

8 Easy Steps

DAY 1 Step 1 Dilute 1ºHu Ab in Reagent 1 Ab Primer

Day 2 Step 1 Prepare slides Deparaffinize & H₂O₂ Block

Day 2 Step 2 Add Reagent 2 Quenching Buffer to Hu-Ab /Reagent 1 Mix

Day 2 Step 3 Add Reagent 3: Hu Blocking A 30min @ RT

Day 2 Step 4 Add Reagent 4: Hu Blocking B 5min @ RT

Day 2 Step 5 Add Mix from Step 2 30 to 120min @ RT

Day 2 Step 6 Add Reagent 5: Hu HRP Polymer 15min @ RT

Day 2 Step 7 Add Reagent 6A&6B: DAB 5min @ RT

**Table 2** Background Screen of Klear Human on Multiple Human Tissues

| Tissue | Background with 1 Hour Detection | | Background with 2 Hour Detection | |
|---|---|---|---|---|
| | HIER | No HIER | HIER | No HIER |
| **Appendix** | - (P+) | - (P+) | - (P++) | - (P++) |
| **Breast** | - | - | - | - |
| **Breast Cancer** | - | - | - | - |
| **Colon Cancer** | - | - | - | - |
| **Esophagus** | - | - | - (P+/-) | - (P+/-) |
| **Heart** | - (P+) | - | +/- | +/- |
| **Hodgkin's Lymphoma** | - (P+/-) | - | | |
| **Kidney** | - | - | +/- (P+/-) | +/- (P+/-) |
| **Liver** | - | - | - | - |
| **Lung** | - | - | +* | +* |
| **Lymph node** | - | - | - | - |
| **Neurofibroma** | - | - | - | - |
| **Placenta** | - | - | - (P+/-) | - (P+/-) |
| **Prostate Cancer 1** | - | - | - | - |
| **Prostate Cancer 2** | - | - | - | - |
| **Skin** | - | - | - | - |
| **Small Intestine** | - | - | - (P+/-) | - (P+/-) |
| **Spleen** | - | - | +/- | +/- |
| **Tonsil** | - | - | - (P+/-) | - (P+/-) |
| **Thyroid Gland** | - (P+/-) | - (P+/-) | | |
| **Uterine Fibroids** | - | - | - | - |

* Tissue is dirty. P: Background in plasma. See figure 4.

**Figure 2a Human Breast Cancer Tissue Array**



Hu anti-Ck18/19      Ms anti-Ck18      Ms anti-Ck19

**Figure 2b Human Colon Cancer Tissue Array**
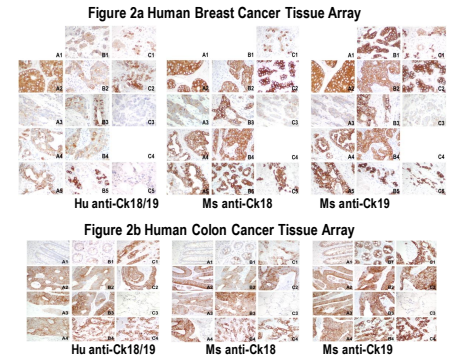


Hu anti-Ck18/19      Ms anti-Ck18      Ms anti-Ck19

**Figure 2a and 2b** contain three panels of breast (2a) and colon (2b) tissue array. The first panel stained with Hu anti-Ck18/19, the second panel stained with Ms anti-Ck18, and the third panel stained with Ms anti-Ck19. The results show different staining intensity among the three primaries with greatest differences in breast cancer tissues. This difference may be due to the 3 antibodies having different antigenic sites for CK18/19 proteins.
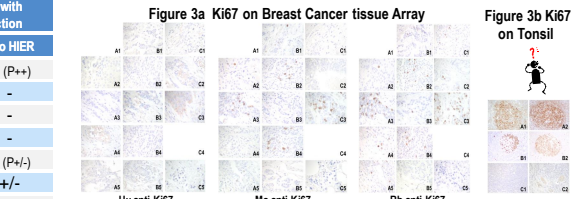
**Figure 3a Ki67 on Breast Cancer tissue Array**



Hu anti-Ki67      Ms anti-Ki67      Rb anti-Ki67

**Figure 3b Ki67 on Tonsil**



**Figure 3a and 3b** show Ki67 expression pattern using a human, mouse, and rabbit anti-Ki67. Fig3a shows Hu anti-Ki67 produced a cytoplasmic stain (panel-1) while both the Ms anti-Ki67 (panel-2) and Rb anti-Ki67 (panel-3) produced the predicted nuclear stain for Ki67 . The same result was produced in colon cancer tissue array for the three primaries (data not shown). Fig 3b shows tonsil with Hu anti-Ki67(A1&A2), Ms anti-Ki67 (B1), Rb anti-Ki67 (B2), and no primary control for Klear Human (C1&C2). Again the Hu anti-Ki67 gave cytoplasmic staining, however this time it was easier to see that the same cells were positive that were positive for the Ms and Rb Ki67 antibodies.
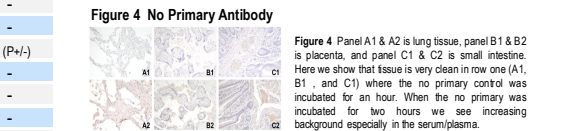
**Figure 4 No Primary Antibody**



**Figure 4** Panel A1 & A2 is lung tissue, panel B1 & B2 is placenta, and panel C1 & C2 is small intestine. Here we show that tissue is very clean in row one (A1, B1 , and C1) where the no primary control was incubated for an hour. When the no primary was incubated for two hours we see increasing background especially in the serum/plasma.

**Conclusion:** This study shows Klear Human kit provides a rapid screen for human primary antibody proposed antigen target in human tissue with minimal background. We show that Hu anti-Hu Ck18/19 was similar to both the Ms anti-Hu Ck18 and Ms anti-Hu Ck19 staining on the colon cancer, but some differences were noted in some breast cancer tissue arrays suggesting it may predict the specificity to tumor subtypes. Finally we show the human anit-Ki67 antibody produced a different staining pattern than predicted. Ki67 further support further support that this kit may assist researchers to assess specificity of their human primary antibody of choice.