Immunohistochemical Staining of B7-H1 (PD-L1) on Paraffin-embedded Slides of Pancreatic Adenocarcinoma Tissue

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Abstract

B7-H1/PD-L1, a member of the B7 family of immune-regulatory cell-surface proteins, plays an important role in the negative regulation of cell-mediated immune responses through its interaction with its receptor, programmed death-1 (PD-1) [1,2]. Overexpression of B7-H1 by tumor cells has been noted in a number of human cancers, including melanoma, glioblastoma, and carcinomas of the lung, breast, colon, ovary, and renal cells, and has been shown to impair anti-tumor T-cell immunity[3–8].

Recently, B7-H1 expression by pancreatic adenocarcinoma tissues has been identified as a potential prognostic marker[9,10]. Additionally, blockade of B7-H1 in a mouse model of pancreatic cancer has been shown to produce an anti-tumor response[11]. These data suggest the importance of B7-H1 as a potential therapeutic target. Anti-B7-H1 blockade antibodies are therefore being tested in clinical trials for multiple human solid tumors including melanoma and cancers of lung, colon, kidney, stomach and pancreas[12].

In order to eventually be able to identify the patients who will benefit from B7-H1 targeting therapies, it is critical to investigate the correlation between expression and localization of B7-H1 and patient response to treatment with B7-H1 blockade antibodies. Examining the expression of B7-H1 in human pancreatic adenocarcinoma tissues through immunohistochemistry will give a better understanding of how this co-inhibitory signaling molecule contributes to the suppression of

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antitumor immunity in the tumor's microenvironment. The anti-B7-H1 monoclonal antibody (clone 5H1) developed by Chen and coworkers has been shown to produce reliable staining results in cryosections of multiple types of human neoplastic tissues[4,8], but staining on paraffin-embedded slides had been a challenge until recently[13–18]. We have developed the B7-H1 staining protocol for paraffin-embedded slides of pancreatic adenocarcinoma tissues. The B7-H1 staining protocol described here produces consistent membranous and cytoplasmic staining of B7-H1 with little background.

Keywords

Cancer Biology; Issue 71; Medicine; Immunology; Biochemistry; Molecular Biology; Cellular Biology; Chemistry; Oncology; immunohistochemistry; B7-H1 (PD-L1); pancreatic adenocarcinoma; pancreatic cancer; pancreas; tumor; T-cell immunity; cancer

Protocol

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1. De-parafinization

1. Bake slides at 55 °C for 20 min.
2. In a chemical hood, immerse slides in xylene for 10 min.
3. Immerse slides in fresh xylene for 10 min.
4. Immerse slides in 100% ethanol for 5 min.
5. Immerse slides in fresh 100% ethanol for 5 min.

6. Immerse slides in 95% ethanol for 5 min.
7. Immerse slides in 80% ethanol for 5 min.
8. Immerse slides in deionized water for 5 min.

2. Antigen Retrieval

1. Immerse slides in Tris-EDTA buffer (pH 9.0) and heat in a pressure cooker to 125 °C for 30 sec, then 90 °C for 10 sec.
2. Let slides cool for 60 min.
3. Immerse slides in TBST for 5 min; repeat twice.

3. Staining

1. Wipe off solutions around the tissue on the slides. Mark a circle around the tissue with a hydrophobic pen.
2. Place the slides in a humidity chamber and avoid the drying of the tissue on the slide throughout the entire staining process. Place one drop (approximately 200 μl or more to cover the tissue area completely) of peroxidase block within the hydrophobic circle on the slide and incubate slides in peroxidase block for 5 min.
3. Rinse slides with TBST, then place in TBST for 5 min.
4. Place one drop of BLOCK ACE blocking buffer on each slide and incubate for 15 min.
5. Rinse slides with TBST, then place in TBST for 5 min.
6. Place one drop of Avidin solution on each slide and incubate for 15 min.
7. Rinse slides with TBST, then place in TBST for 5 min.
8. Place one drop of Biotin solution on each slide and incubate for 15 min.
9. Rinse slides with TBST, then place in TBST for 5 min.
10. Incubate slides with 1:1,000 dilution of mouse anti-B7-H1 monoclonal antibody (clone 5H1) or a mouse IgG1 κ isotype control in 200 μl TBST at 4 °C overnight for 22 hr +/− 1 hr. The final antibody concentration in TBST is 2.57 μg/ml.
11. Rinse slides with TBST, then place in TBST for 5 min. Repeat twice.
12. Incubate slides with a biotin conjugated rat anti-mouse IgG1 secondary antibody at a 1:500 concentration (in 200 μl 2% goat serum solution) for 30 min.
13. Rinse slides with TBST, then place in TBST for 5 min. Repeat twice.
14. Place one drop of Streptavidin-Biotin Complex (must be prepared at least 30 min prior to use) on each slide and incubate for 15 min.
15. Rinse slides with TBST, then place in TBST for 5 min. Repeat twice.
16. Place one drop of Amplification Reagent (diluted 2:1 with TBST) on each slide and incubate for 4 min.
17. Rinse slides with TBST, then place in TBST for 5 min. Repeat twice.
18. Place one drop of Streptavidin-HRP on each slide and incubate for 15 min.
19. Rinse slides with TBST, then place in TBST for 5 min. Repeat twice.

20. Place one drop of 3,3-Diaminobenzidine (DAB) substrate and chromogen on each slide and develop the color for 2 min.
21. Immediately after the color development, wash slides with ddH_2O for 1–2 min.
22. Incubate slides with one drop of hematoxylin for 90 sec.
23. Wash with ddH_2O for 1–2 min.
24. Wash with soapy tap water for 1–2 min.
25. Wash with ddH_2O for 1–2 min.

4. Dehydration and Mounting

1. Immerse the slides in 80% ethanol for 3 min.
2. Immerse the slides in 95% ethanol for 3 min.
3. Immerse the slides in 100% ethanol for 5 min. Repeat.
4. Immerse the slides in xylene for 5 min. Repeat.
5. Add one drop of Cytoseal 60 to the slide and place the coverslip.

Table 1 lists the reagents used in the above described procedures.

Representative Results

A successful immunohistochemical staining of B7-H1 will demonstrate the heterogeneous expression of B7-H1 (brown signals DAB color development) in pancreatic adenocarcinoma. Some pancreatic adenocarcinoma cells have predominantly membranous expression of B7-H1 (Figure 1A) whereas others have either predominantly cytoplasmic expression of B7-H1 or little expression of B7-H1 (Figures 2 and 3). Normal pancreatic duct epithelium expresses little B7-H1 (Figure 4). Staining with a mouse IgG1 κ isotype control antibody instead of the anti-B7-H1 antibody shows little background staining (Figure 1B).

Discussion

Immunohistochemical staining of B7-H1 on cryosections has been reported in prior studies for a variety of cancers[3,4]. However, clinical tumor samples usually are available only in the form of paraffin-embedded blocks. The 5H1 monoclonal antibody has more recently been successfully applied to the staining of paraffin embedded tumor tissues including renal cell carcinoma, cervical carcinoma, bladder cancer and melanoma[13–18]. B7-H1 staining on paraffin-embedded[9,19] or cryostat[20] sections of pancreatic adenocarcinoma tissues with other monoclonal antibodies has also been reported. Here, we have shown successful staining of paraffin-embedded pancreatic adenocarcinoma tissues with the 5H1 monoclonal antibody to B7-H1. The expression of B7-H1 in pancreatic adenocarcinoma is heterogeneous, similar to that in many other types of malignancies such as melanoma[4,21]. Both membranous and cytoplasmic expression of B7-H1 are observed with adenocarcinoma cells (Figures 1 and 2). Membranous expression of B7-H1 is consistent with its biological function. The role of cytoplasmic B7-H1 in neoplastic tissues remains to be explored. Normal pancreatic duct epithelial or acinar cells do not express B7-H1 (Figure 4).

All the steps in this protocol take place at room temperature, except for the overnight incubation at 4 °C. Antigen retrieval conditions used in this B7-H1 staining procedure were not reported in the aforementioned publications[9,19]. The amplification step was also not employed by these studies. We found that the critical steps in this protocol include the antigen retrieval, the overnight incubation with the primary antibody, the amplification, and

the DAB color development. The dilution of the primary antibody might need to be titrated slightly for each different batch of 5H1. Amplification has been kept exactly at 4 min with a diluted amplification reagent. These modifications give sufficient amplification of positive signals without increasing background. The development time is typically 2 min, but we routinely monitor the development under a microscope to ensure optimal staining with minimal background. With each round of staining, we include one slide from a previous batch and one slide of tonsil tissue as positive controls. Paracortical staining of B7-H1 in tonsil has been previously described[4]. If the background is high or if it takes more than 5 min to develop typical signals in these control slides, we recommend replacing the reagents for the critical steps and checking all staining steps.

The use of this staining technique allows for qualitative and semi-quantitative analysis of B7-H1 expression and localization on neoplastic pancreatic adenocarcinoma cells. With modification of the dilution of the primary antibody, the length and dilution of the amplification step, and the time of color development, this protocol may be applied to other tissue types. We have modified this protocol and used it for breast cancer and sarcoma tissues. As B7-H1 and its binding to PD-1 provide a major tumor-induced immunosuppressive signal in the tumor's microenvironment, this staining technique will facilitate our understanding of the role of the tumor's microenvironment in tumor initiation, progression and anti-tumor immune response.

Acknowledgments

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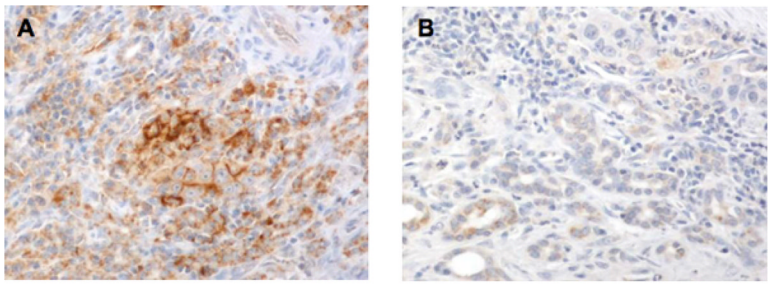

Figure 1.

A. Immunohistochemical staining with the anti-B7-H1, clone 5H1 antibody shows membranous expression on some pancreatic adenocarcinoma cells. **B.** Immunohistochemical staining with the isotype control IgG1 shows little DAB color development on the same pancreatic adenocarcinoma tissue.

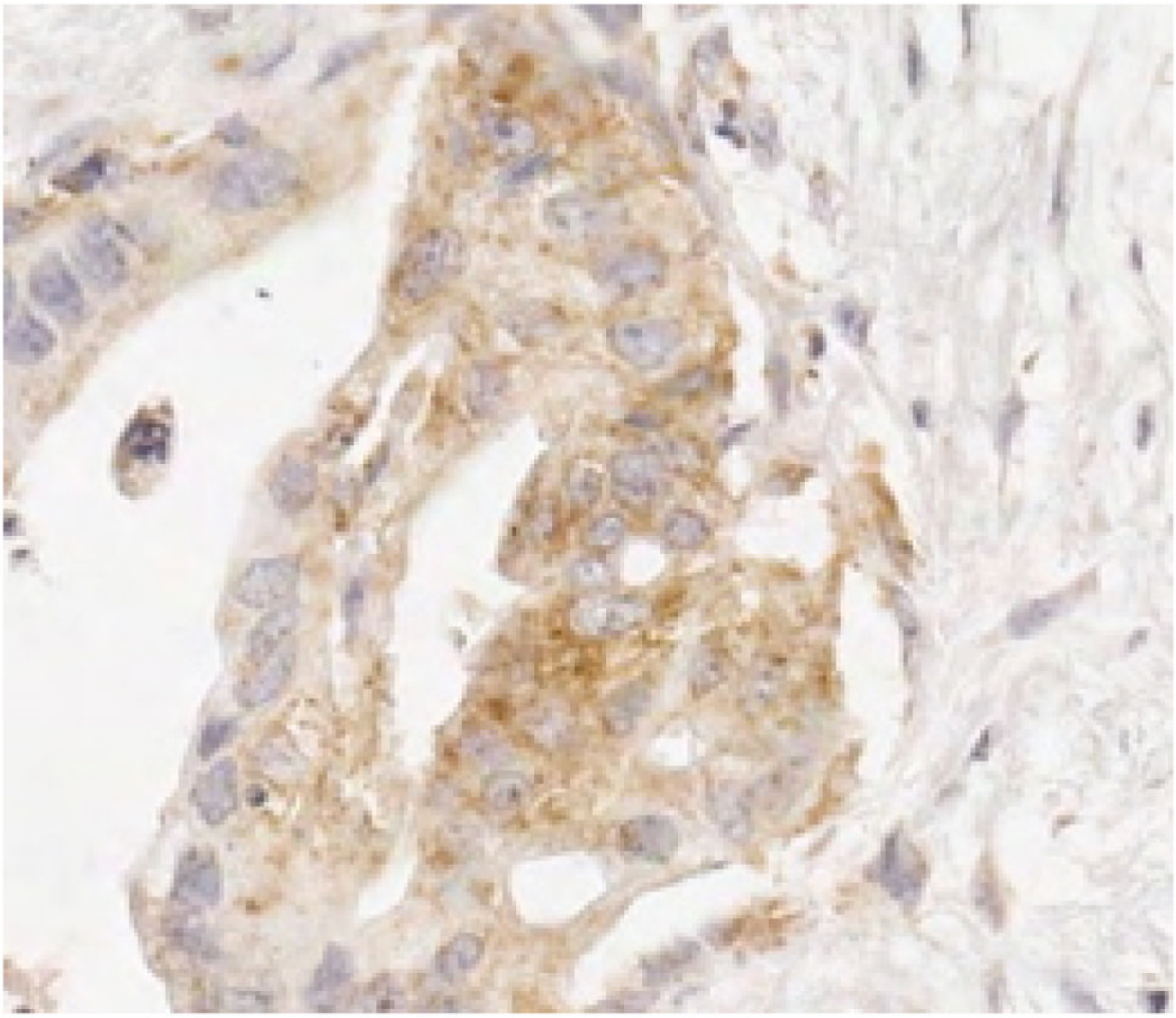

Figure 2.
B7-H1 expression in the cytoplasm of pancreatic adenocarcinoma cells.

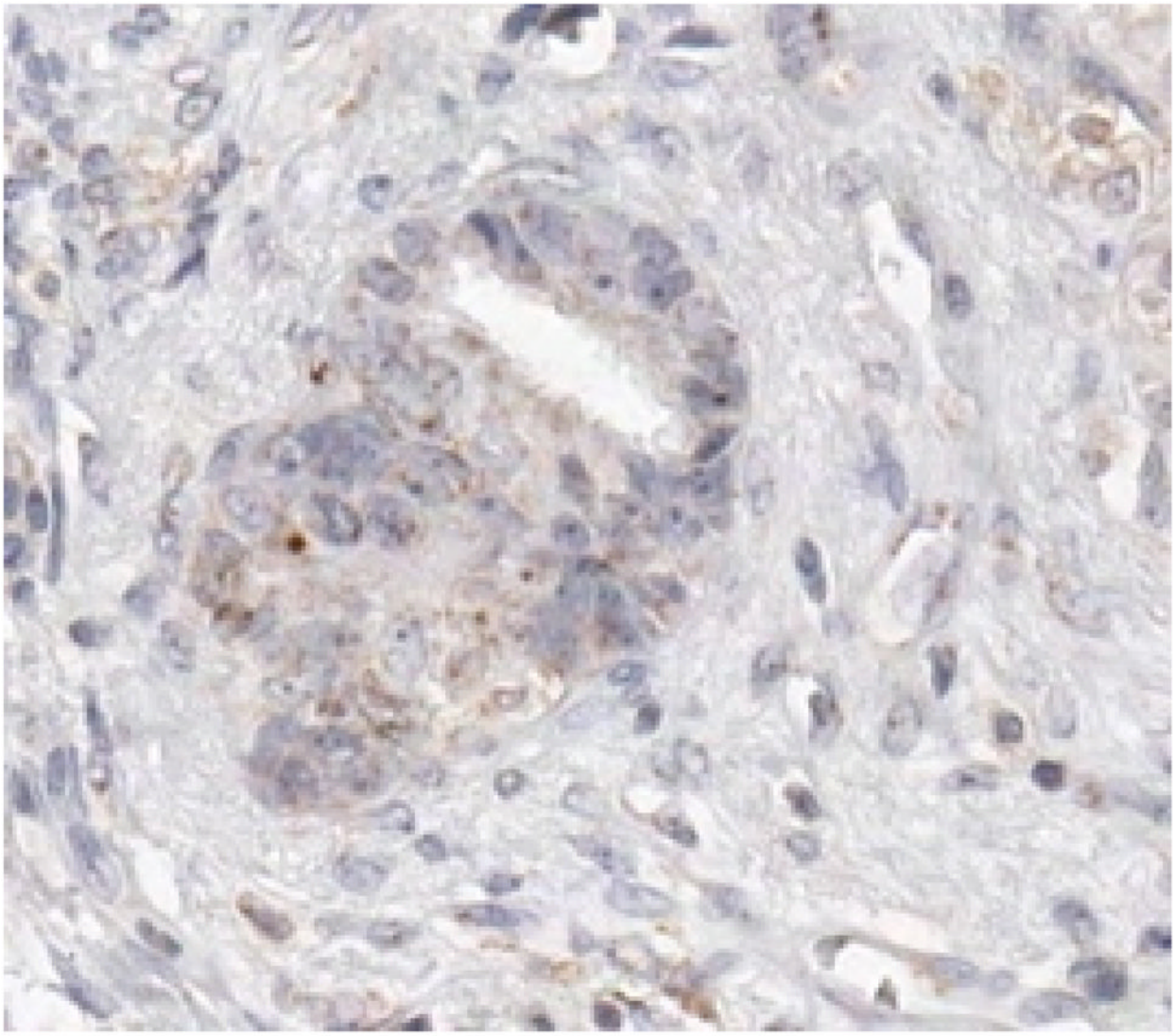

Figure 3.
Some pancreatic adenocarcinoma cells express little B7-H1.

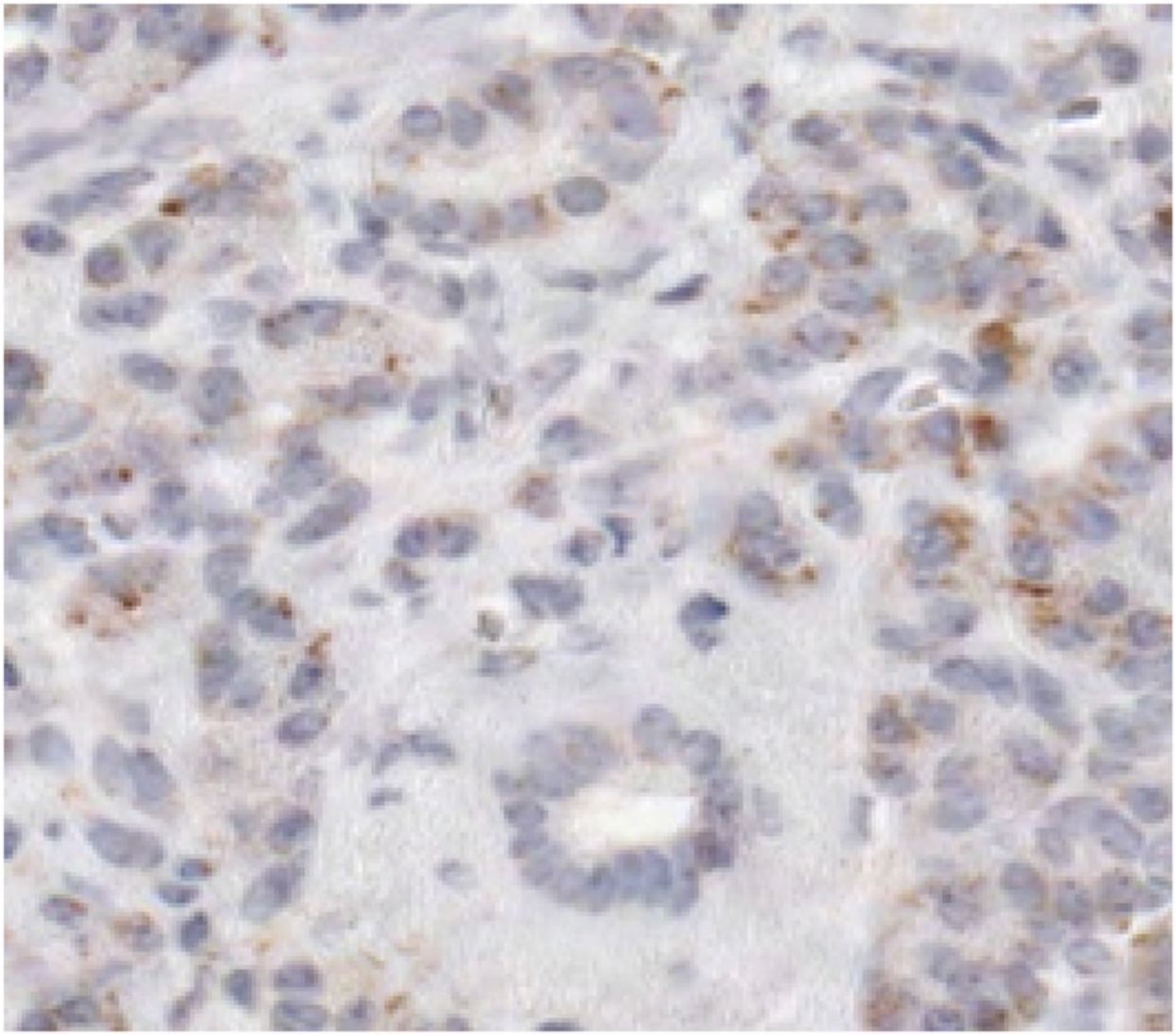

Figure 4.
B7-H1 expression on normal pancreatic ductal epithelium.

Table 1

Reagents and equipment.

Name	Company	Catalog Number	Comments
CSA System	Dako	K1500	Includes the following reagents mentioned in the protocol: peroxidase block, streptavidin-biotin complex, amplification reagent, streptavidin-HRP, and DAB substrate and chromogen
Avidin/Biotin Blocking Kit	Vector	SP-2001	
Block ACE	Serotec	BUF029	
Biotin anti-mouse IgG1	BD	553441	
Mouse IgG1 K Isotype Control	eBioscience	14-4714-85	
TBS(Tris-buffered saline), 10X	Cellgro	46-012-CM	10xTBS contains 80 g/L NaCl and 24.2 g/L Tirs; Diluted to 1X with ddH2O for washes;
Tween 20	Sigma-Aldrich	P1379	1 ml added to 1 L of 1X TBS to make TBST
Target Retrieval, 20x	Celerus Wave	014-3000-000	Diluted to 1x with ddH_2O
190 Proof Ethyl Alcohol	Pharmco-Aaper	111000190	
200 Proof Ethyl Alcohol	Pharmo-Aaper	111000200	
Xylene	VWR	95057-827	
Hematoxylin	Richard-Allan Scientific	72804	
Cytoseal 60	Richard-Allan Scientific	8310-4	
Coverslips	Corning	2940-245	
Humidity chamber	Sigma	H6644	