Programming and Validation of an Autostainer

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Abstract

Histology performed by Cal Poly students for research projects and labs are currently performed by hand using a dipping technique. This technique is time consuming, expensive, imprecise, and often unrepeatable. Central Coast Pathology donated a Dako Autostainer to the Biomedical Engineering Department that can perform immunohistological stains of up to 48 samples simultaneously using tightly controlled reagent volumes and incubation times. Protocols are programmed into the machine and can be repeated under the same conditions every time with minimal preparation and exposure to potentially dangerous reagents. The autostainer was repaired and validated by comparing PECAM & BBI stains performed on human umbilical vascular endothelial cells (HUVEC) with the autostainer. HUVEC cells contain the extracellular antigen that PECAM binds to and DNA which BBI interacts with. The stain procedure was a success as the images of the stained HUVEC contained fluorescent portions from both PECAM and BBI. Finally a guide was developed to assist students to successfully create and run their own protocols using the now functional autostainer.

Introduction

Background

The basic principle of histology is to add chemicals or antibodies to a sample of tissue that will color or visually differentiate specific physiological features from the rest of the sample. Histology allows researchers to visualize and distinguish features easily from cross-sections of tissue. Untreated tissue samples are nearly invisible with light microscopy since they need to be thin enough for light to pass through them. The high water concentration of tissue and lack of pigment of many cell structures makes them even harder to see if untreated. There are countless methods used to color nearly any feature in a tissue and immunohistology is incredibly specific.

The two main types of histology are traditional histology, or histochemical staining, and immunohistochemical staining. Histochemical staining uses chemical interactions between various reagents and the targeted tissue feature. This method works well and has been used for many decades. Immunohistochemistry is a procedure used to examine structure and protein expression in tissue samples using antibodies. Antibodies are much more specific than histochemical stains since they will only bind to a single antigen. Researchers can visualize any type of tissue by attaching a colored or fluorescent molecule to the antibodies.

Before a tissue can be treated for either traditional histological staining or immunohistochemical staining it must first be fixed, dehydrated, cleared, and embedded for sectioning. The purpose of fixing the tissue sample is to flush out water and deactivate any enzymatic activity. Continued enzyme activity after the sample has been removed can lead to undesired protein expression and sample degradation (Dabbs, 19). The fixative will also neutralize bacteria and fungi in the tissue samples (Dabbs, 19). Excess water in the tissue will cause the cell to be squishy and less rigid, resulting in a sample that will not section properly (Dabbs, 19). Ideally the fixative should not alter the tissue,

remove antigen binding sites, or cause excessive diffusion and displacement of tissue features. Ethanol and formalin (watered down formaldehyde) are the two most common reagents used for tissue fixation (Dabbs, 19). Ethanol and formalin both preserve the tissue but ethanol causes many features such as DNA and protein to coagulate, denature, or be flushed away in the alcohol or water (Dabbs, 19). Formalin, on the other hand, creates cross linked structures between the proteins in the tissue that hold the tissue features in place, preserving antigen binding sites, while still maintaining the general protein conformation(Dabbs, 19). Formalin is also cheaper, preserves the tissue for longer, and sterilizes the tissue (Dabbs, 19).

Once the tissue is fixed it is dehydrated with the use of ethanol and cleared with xylene or toluene (Dabbs, 19). Ethanol flushes the water out, while the clearing agent flushes the excess ethanol from the tissue (Dabbs, 19). Next paraffin is added to the tissue to add structural support, replacing the clearing agent (Dabbs, 19). Finally the processed tissue is embedded in a plastic epoxy that is a little harder than paraffin (Dabbs, 19). A harder plastic allows for smaller cross sections to be taken from the sample on the microtome. Thinner samples will allow for narrower, more precise sections of the tissue to be analyzed. Tissue sample thickness will also affect the diffusion rates of reagents during the staining procedure (Hansen, 4). This will affect the development of the stain and the amount of light that can pass through unstained features in the tissue when viewed under the microscope (Hansen, 4). The thin tissue slices are then mounted on glass slides with an acrylic adhesive (Dabbs, 19).

Samples fixed in formalin and encased in paraffin are referred to as formalin-fixed paraffin encased (PFFE) tissue samples and can keep at room temperature for decades without degrading (Shi, 13-14). They can be used for traditional histology protocols but can also be boiled to be used for immunohistochemical stains as well (Shi, 13-14). Boiling removes the PFFE samples removes the paraffin and undoes the cross bridging of formalin, returning the tissue to aqueous solution (Shi, 13-14).

Most if not all of the tissue features are retained. This is particularly important for immunohistochemical staining as this procedure allows the antibodies to penetrate the tissue and bind to its antigen. This procedure is referred to as antigen retrieval (AR). Some current studies have even shown that DNA and RNA can be extracted intact from PFFE (Shi, 13-14). It has also been shown that microwaving the PFFE sample increases the effectiveness of the AR procedure (Leong).

Histological staining is a complex procedure that requires the addition of many reagents over a long time course. Precision in the concentration and incubation times of each of these reagents is directly correlated to the quality of the developed tissue sample. There are six main sources of artifacts in traditional staining technique: displaced tissue from the surgery; fixation errors; over staining; artifacts from the sectioning process; insufficient staining; and incorrect staining (Dettmeyer).

The basic procedure of a traditional histological stain is to reverse the embedding procedure to return the tissue to an aqueous environment (Ross, 2). First xylene or toluene is added to remove the paraffin, then ethanol is used to flush out the xylene and finally the samples are slowly rehydrated (Ross, 2). Aqueous dyes can be added at this point (Ross, 2). If the dies are non-polar then the samples are slowly treated with ethanol first (Ross, 2). Some dyes require special pH and temperature conditions for proper binding of the dye (Ross, 2). The samples are gradually brought to the required condition, dyed, then brought to the optimal condition for the next dye (Ross, 2). Once the staining procedure is complete then the tissues are treated with ethanol, then xylene or toluene again to remove water (Ross, 2). Finally the tissues are set on a slide for viewing or archiving (Ross, 2).

Immunohistochemical histology is a little different but the basic principle is the same. Immunohistochemical histology relies on the highly specific binding between an antibody and its antigen. Antibodies are made up of two pairs of two chains of protein that form a binding side and an inert side (Figure 1). The active binding side has two arms, the tips of which form complex

conformational structures that allow the antibody to bind to specific antigens. Monoclonal antibodies produced in a lab are all of the same type and will all only bind to a single antigen while polyclonal antibodies will bind to many antigens and are not as clinically useful (Figure 2). This property can be used to detect nearly any type of molecule or structure in a tissue sample.

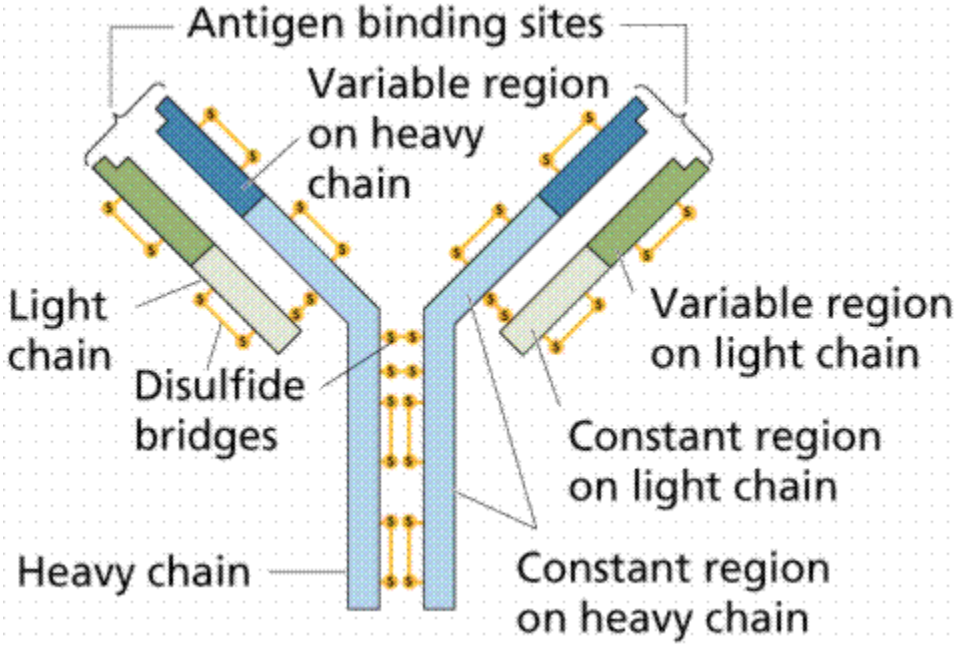


Figure 1: Diagram of an antibody (Farabee).

Antibodies are extremely small and not visible under light microscope without some modifications. Fluorescent molecules, colored molecules such as gold, or enzymes that can carry out a reaction that will cause color change can all be immobilized onto the inert side of the antibody. These labels or flags allow the researcher to visualize the tissue structure the antibody bound to. There are countless labeling molecules. This labeling process is somewhat difficult and obtaining monoclonal antibodies to a rare or hard to isolate antigen can be tedious and expensive. Often a sandwich, or indirect, method is used with antibodies whose antigen is the inactive side of other antibodies (Figure 3) (Dako Pathology). A single type of labeled monoclonal anti-antibody can be used to bind and distinguish

any other antibody since all antibodies have the heavy chain (except in the active site at the tip of the Y arms). Another method of tagging the antibodies is with fluorescent molecules. As with the methods outlined above these can also be tagged directly or indirectly (Figure 3). Direct methods of immunohistology take less time to prepare but can be costly (Dako "Pathology", 61). Indirect methods allow for amplification of low concentrations since many tagged antibodies can bind to a single primary antibody, but can cross react with one another and add noise to the sample (Dako "Pathology", 61).

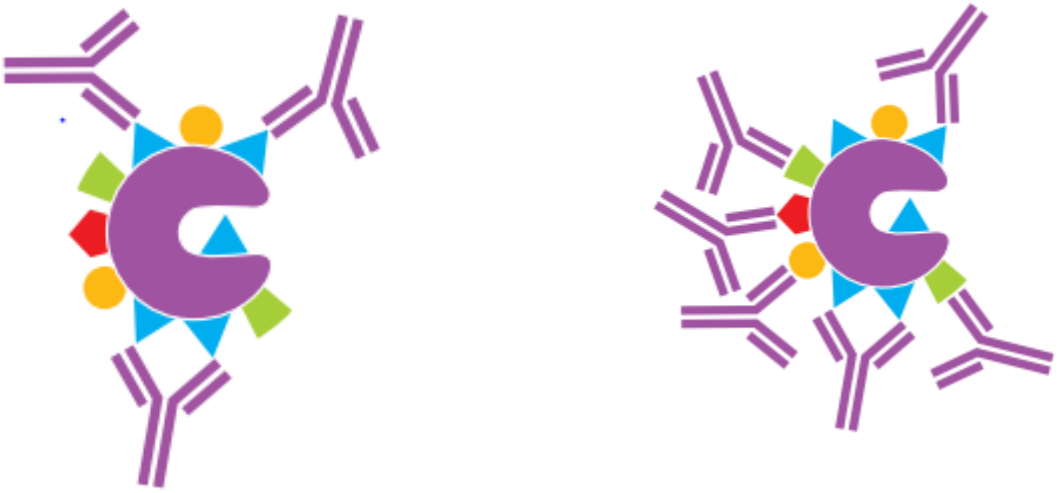


Figure 2: Left is a diagram of how monoclonal antibodies attach to their antigen (and no other antigen) on a cell while on the right polyclonal antibodies bind non-specifically (Dako "Pathology", 3 and 4).

Because antibodies are so specific, they can even be used to perform accurate measurements of concentration of their antigen by measuring the absorbance of the colored region if the staining protocol is repeated exactly between each sample and control. If the region has a higher concentration of antigen it will be much more colored and a higher measurable absorbance (Taylor and Cote, 3-11).

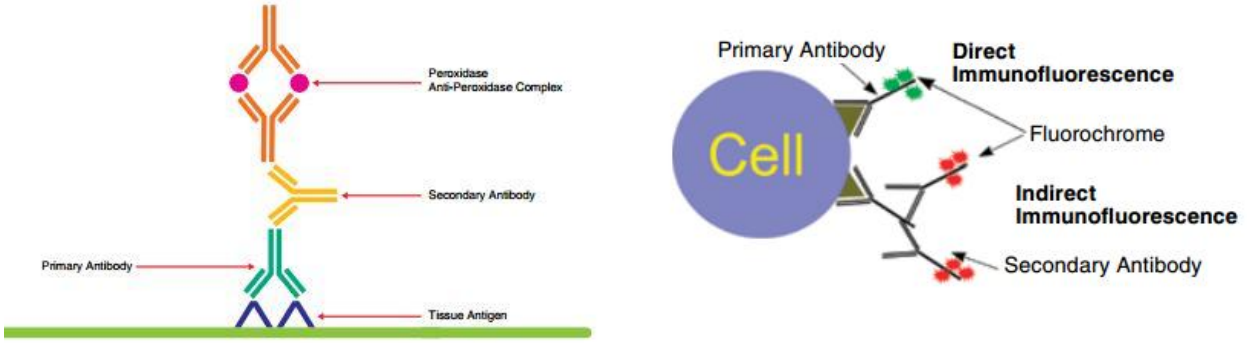


Figure 3: Left shows the indirect, or sandwich, method of immunohistology using an enzymatic reaction, the right shows direct and indirect method of immunohistology using fluorescently tagged antibodies (Dako "Pathology", 57 and 61).

The current method of performing histology at Cal Poly is the basket dip method. This method involves placing tissue sample slides in a metal slide holding basket (Figure 4) and dipping it in baths of reagents for the required incubation time (Figure 5). This method is highly inefficient, imprecise, wastes reagent, and results in lost tissue samples.

Figure 4: The metal slide baskets currently used for performing histology at Cal Poly. Note the orientation of the slides (perpendicular to the reagent, causing shear) and the metal spring handle that can easily fall off while moving the slides.

Students are inexperienced with performing histology protocols and may even be performing histology for the first time, learning as they go. The fluid nature of student researchers reduces the number of qualified and experienced students. These students are likely to make mistakes on long staining protocols that require precise timing and repetitive steps. It is easy to lose place in a repetitive protocol and some steps may be skipped or repeated. Small user errors, even minute changes in incubation times, can also result in inconsistent stains. Inconsistent stains are all developed a little bit differently, making comparison of different tissue samples difficult or impossible. If only a few tissue samples are available then this can ruin an experiment (Deckert). These inconsistencies in staining can cause false positive and false negative stains (Deckert).

Histology done by hand requires large baths of reagents and lots of buffer solution. Many of these reagents are quite expensive and are a limiting factor for how many samples a student is able to examine for their project. Furthermore, many of the reagents are dangerous to handle. Students spill these reagents often while pouring the reagents into the baths and while lowering the slide baskets into the baths. Also, many reagents are spilled after the protocol is complete and the students pour the bath into waste jars. This both wastes expensive reagent and exposes the students to potentially dangerous chemicals.

Figure 5: The reagent baths used for the basket dip method. Note the large volumes of reagent used and the ease of accidentally dipping the slides into the wrong reagent bath (Hasumi).

Many staining protocols are very lengthy and require long incubation times. This presents many problems. Because lab classes at Cal Poly are only three hours long this greatly reduces the number of staining procedures that can be performed in a class. Research groups doing histology for projects do

not have enough free time to perform these length staining procedures. The longer a staining procedure is the more chances there are for user error, especially as students become restless, bored, and lose ambition in the repetitive protocol.

If the person performing the protocol gets distracted the samples can be destroyed. Some of the reagents used will destroy the tissue sample if left for too long and if the samples are left to dry for too long they can dry out and deteriorate. Also, histology is currently performed with histology baskets and a dipping technique. If this procedure is done recklessly the samples can fall off the slides and be lost in the reagent bath. Since the slides are held perpendicular to the water surface they are subject to a large amount of shear when raised and lowered from the bath (Figure 4). The handles on the basket are not fixed the basket firmly so that the lid of the reagent bath can be left on during incubation (Figure 4 and 5). If the student squeezes the handle when picking up the slide basket then the basket will fall. This often results in spilled reagent and broken slides.

Many protocols require the use of reagents that degrade quickly in the presence of light. These protocols would usually be performed in darkness, increasing the likelihood of making mistakes.

Plan

Recently a Dako Autostainer was graciously donated to the Cal Poly's College of Biomedical Engineering. This device can stain up to 48 slides of samples simultaneously using the exact same protocol for each slide with full reproducibility. The goal of this project is to address the many challenges of the current histology methods by setting up, programming, and quantitatively validating the resultant stains from the autostainer.

Figure 6: The Dako autostainer with proprietary software (University of Florida).

The autostainer will save money on reagents and buffer because it uses much less volume. Because the reagents are dropped from a small height directly over the tissue sample only small amounts of reagent are required to coat the slide. The autostainer also has a blower that it uses to remove buffer from the slide before the next reagent is added. This prevents the next reagent from being watered down and therefore requires even less reagent to be used. Exposure to the reagents will be minimized because the student does not need to constantly handle the slides or reagent baths. The reagents are added once in the beginning of the protocol in small sealable tubes. The staining area of the machine is also encased in a hood so that volatile reagents do not leak out into the lab. The waste is handled internally and the autostainer differentiates the hazardous and non-hazardous waste into separate carboys.

The autostainer also prevents tissue sample loss. Samples cannot be lost due to drying out because it has a built in hydration procedure where it always adds buffer or reagent to the sample during and after the staining procedure. The only problem with this is that if the autostainer is left unattended for too long after a protocol is run to completion it can overflow the waste carboys with fluid. The protocol will always be run as programmed so as long as the reagents are prepared correctly (correct concentration, volumes, and placement into machine) then the correct amount of reagent will

be added to each slide and incubation times will be precise. The slides are held horizontally on a tray and reagents are dripped gently from a small height above, minimizing shear. This minimizes shear on the tissue samples in the autostainer so samples should not fall off the slides.

The autostainer will reduce or completely eliminate user error during the staining protocol. If the protocol is robust and programmed into the autostainer correctly then tissue samples should receive the same treatment each time with the correct reagent order and incubation time. User error can still enter into the protocol in the preparation of the tissue sample and in reagent preparation (incorrect concentration or volume). Furthermore, since each tissue sample will receive the same treatment they should all have the same development, allowing them to be compared to one another. This is referred to as a true test since all of the samples should be able to be compared to one another (Taylor). If it is a true test then with the use of proper controls the staining protocols should yield significant, definitive results every time (see Table II below).

The autostainer will allow students and researchers to perform a wider variety of lengthy and more difficult protocols since the autostainer can run with little to no supervision once the protocol has been started. Longer stains can be simply left to run and analysis can be done on another lab day. Since the students will not have to spend the lab period performing the stains they perform a different lab experiment to learn something else or even spend time to develop refinements to existing protocols. Because the autostainer will automatically keep the slides hydrated with buffer after the protocol is completed the samples can even be left overnight and collected the next day. The only caveat to this, as mention earlier, is that the waste carboys can overflow with buffer solution if left unattended for too long.

The autostainer is not without its own shortcomings, however. Tissue samples that are mounted too close to the label end of the slides often get stained incorrectly (Colley). Also the machine

at Cal Poly is older and runs on an older version of the Dako software. The newer machines have a newer version of the software that dramatically decreases the run time of each stain by making changes to the pipette arm path (Colley). There is a pretty steep learning curve just to be able to operate the machine, let alone program new protocols into it.

Finally a basic guide or protocol was developed to aid students in using the autostainer without my help (Appendix D). As students use the machine the protocol can be refined.

Methods

Experimental

Repairing the Autostainer

The entire autostainer was received intact and the company who donated the machine claimed it operates fine. After inspecting the machine a few missing and broken pieces were found and replaced. Two Nalgene bottle caps with tubing and filters attached were required to connect the DI water and buffer solution pumps to the DI water and buffer solution carboys (see Figure 7, below); a quick disconnect tubing connection for the wastewater output was replaced (see Figure 8, below); a stopcock in the z-head assembly was replaced (see Figure 9, below).

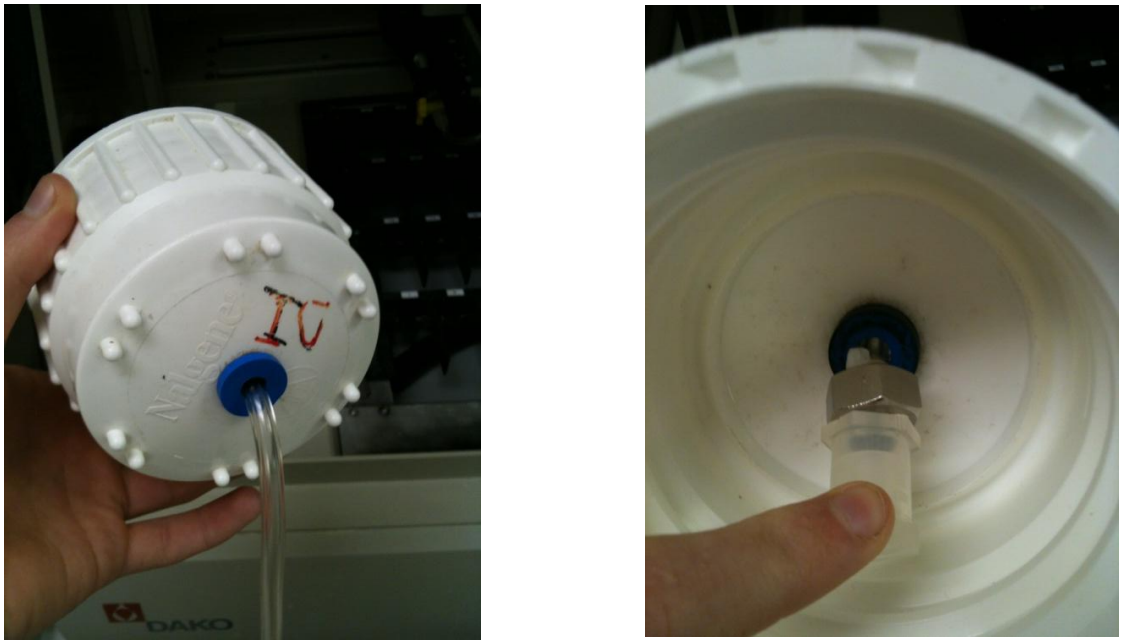


Figure 7: Images of the carboy tops assembly. Left image has the tubing going into the DI water input, right image shows the filter and locking assembly on the inside of the cap.

Once the machine was repaired a cleaning protocol was performed to test if the servos were functional.

Figure 8: The quick connect valves on the waste tubing. The right one is intact while the left is broken.

Proving Autostainer is Functional

The autostainer has a cleaning routine programmed into the software. The protocol involves rinsing the slide racks with a small volume of DAB Away cleaning solution that is placed in the reagent rack (see Appendix A for running self-cleaning protocol) and was used to test if the pipette head servo, DI water pump, and buffer solution pump are operational without wasting reagent or having to program a stain. DAB cleaning solution was replaced with DI water. The machine performed the operation with no problem.

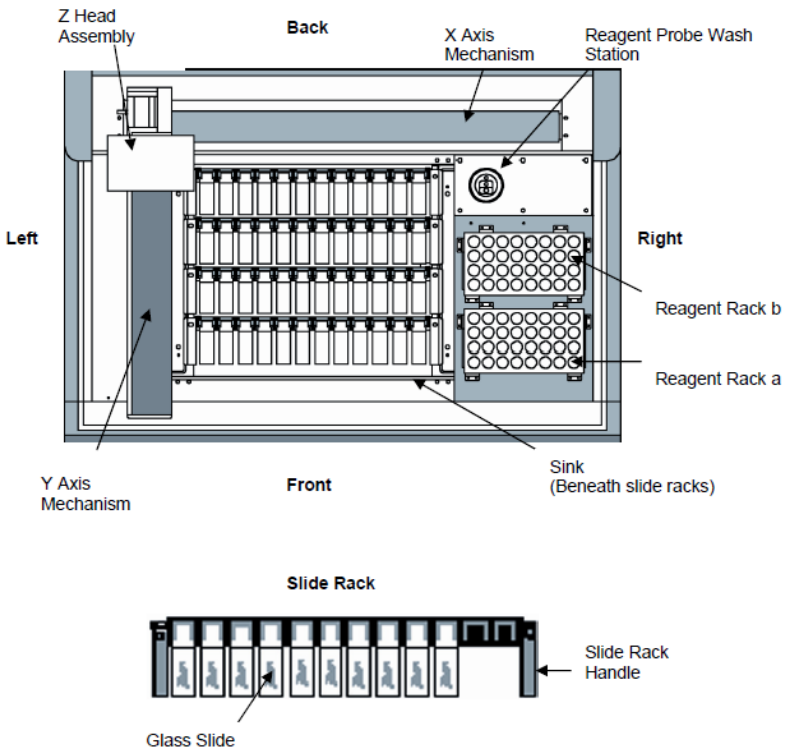


Figure 9: Diagram of the work area of the autostainer (Dako "Dako Autostainer Handbook", 45).

After the missing parts were purchased and installed the machine was given a thorough cleaning using DI water and a brush was performed. Chemical reagents cannot be used on the machine as they could react with staining reagents during use later (Dako "Dako Autostainer Handbook", 97). The reagent racks were soaked in DI water and scrubbed. The sink and exterior were cleaned with paper towels (Figure 9).

Programming Stain Protocols

Once the machine was proven functional and cleaned thoroughly the first stain was performed. Appendix D contains the recommended protocol for programming a stain. The general procedure is to first set the order of steps of the procedure in the "Protocol Outline" screen. Next the reagents for the stain are inserted in the new reagent screen, along with their incubation times. On the slide template

screen up to 48 sample slides can be added and the protocol outline can be assigned to them. The reagents are then chosen for each step of the protocol outline and the protocol can be carried out.

A test stain of endothelial cells was run. A platelet endothelial cell adhesion molecule (PECAM) was targeted along with a counterstain of bisbenzimide (BBI) on a sample of human umbilical vascular endothelial cells (HUVEC). The PECAM molecule, also known as cluster of differentiation 31 (CD31) is found on the outside of endothelial cells while BBI binds between adenosine and thymine bonds in the DNA backbone. The procedure was performed as outlined in Appendix B on a single slide with HUVEC tissue.

Developing Machine Protocol for Students

With the machine functional students can use it for their research. Students who need access to the autostainer should be trained on how to enter a protocol into the machine themselves. A brief set of instructions was written that explains how to turn on the autostainer, program a protocol, and set up their samples and reagents (Appendix D).

Part of developing this protocol included determining the proper drying time of the slide mounting adhesive. The staining run performed by Michael Machado on 3/11/12 failed because many of the coverslips fell off of the slides during the staining procedure. While mounting the coverslips to the slides he noticed that the cells on the coverslips were starting to desicate so he added some PBS onto the cells. The PBS mixed with the toluene mounting adhesive and likely caused improper setting. However, to determine the proper coverslip mounting procedure for the autostainer I ran a test using the same test protocol but replaced all of the reagents with PBS (test procedure is in Appendix C).

Students should also design their staining protocols with the proper controls (Table I) and methods (Table II) to ensure quality results (Taylor and Cote, 14).

Table I: Guide for Selecting Controls in an Immunohistochemical Procedure to Ensure Quality and Differentiable Results (Taylor and Cote, 14)

Type of Control	Antigen (Analyte)	Antibody (Reagent)	Purpose
Positive	Non-patient tissue or cells containing antigen to be detected and quantified Known expected result Fixed-processed in same way as a patient sample Fixed-processed in manner shown to preserve antigen under analysis	Antibody reagent (of the kit) constituted in same way as intended for patient sample	Control of all steps of the analysis Training user for appearance of positive reaction; comparison for semi-quantitation of reaction Validates all steps of analysis, including fixation and processing Validates all steps of analysis, except fixation or processing used by individual laboratory
Negative (specific)	Tissues or cells expected to be negative by antibody (of kit) Processed in same way as patient sample May be portion of patient sample	Antibody reagent (of kit) constituted in same way as intended for patient sample	Detection of unintended antibody cross-reactivity to cells or cellular components
Negative (non-specific)	Patient tissue with components that are the same as tissue to be studied Processed in same way as patient sample	Diluent (as used with antibody) without antibody Antibody not specific for antigen of interest in same diluents as used with kit antibody	Detection of unintended background staining

Table II: Recommendations by the Standardization Ad-Hoc Consensus Committee to Improve the Standardization and Quality of Histological Sections (Yaziji et al).

1	Fix all specimens promptly in 10% neutral buffered formalin.
2	Fix and process resections and core biopsies in an identical manner.
3	Only use 10% neutral phosphate buffered formalin.
4	Note that fixation is 8 to 72 hours for both core biopsies and resections.
5	Use formalin, not alcohol, to fix cytology specimens for ER assay.
6	Use conventional tissue processors to process breast tissue.
7	Ensure that the first formalin containers on the tissue processor are always newly replenished.
8	Ensure that tissue processor fluids do not exceed 37°C.
9	Be sure that paraffin in the tissue processor does not exceed 60°C.
10	Record and document fixation times in your report.
11	Use *in vitro* diagnostic kits that employ clone, 6F11, 1D5, or SP1.
12	Include positive and negative controls with each batch run.
13	Employ a threshold for positive result of 1% positive cells.
14/15	Report semi-quantitation, tabulating the intensity (0, 1+, 2+, 3+) and percent of positive cells.

Organizational

Timeline

12/28/11 – Ordered 2 Tube and Cap Assemblies from Dako.

1/6/12 – Tube and Cap Assemblies received and installed on autostainer. I was going to run the self cleaning protocol to check and see if the pumps were working but noticed that one of the waste tubing connections was broken. Need new connection piece.

1/9/12 Ordered pipe fittings to fix waste line connection.

1/12/12 Received pipe fittings and fixed the waste lines. I attempted to run the self cleaning protocol using only DI water but while the pumps were priming I noticed a leak. I cancelled the protocol.

1/13/12 Investigated leak and found that the source was a broken stopcock in the servo arm.

1/16/12 Ordered new stopcock from Dako.

1/18/12 Received and installed stopcock piece. Ran the self cleaning protocol with only DI water and everything worked smoothly.

2/2/12 Cleaned machine thoroughly and programmed in PECAM & BBI staining procedure.

2/4/12 Ran the PECAM & BBI staining procedure on HUVEC sample with Michael Machado (Appendix B).

3/6/12 Met with Michael Machado and programmed in PECAM, ZO-1, CASPAN stain.

3/11/12 Helped Michael Machado set up autostainer to run his 48 samples (run failed).

4/17/12 Ran cover slip adhesion test (Appendix C).

Materials List

The materials list only includes the parts required for repairing the autostainer. Reagents used for the staining procedures are part of other research projects on campus.

Table III: Abridged Project Materials List

Item	Qty.	Price (USD)	Order Price (USD)	Supplier
Carboy Cap Assembly	2	173.65 ea	404.22	Dako, Item # 992375
Autostainer Stopcock	1	128.70 ea	173.67	Dako, Item # 992640
Polypropylene Quick-Disconnect Tube Coupling, Plug, 1/8 Coupling, for 1/4" Tube ID, without Valve	1	2.41	7.36	McMaster-Carr, Item # 51545K68

Budget

The autostainer was repaired using only the above listed parts. Therefore the total budget of the project was $585.25. The cost of repairing the autostainer will be offset by money saved on reagent and labor costs.

Results

Initial PECAM & BBI Stain of HUVEC Sample

The PECAM and BBI stain of HUVEC tissue sample carried out on 2/4/12 as outlined in Appendix B was a success (Figure 10, next page). The stain appeared as expected with both blue and green staining from the BBI and PECAM, respectively.

The procedure used much less reagent than the original protocol it was adapted from (Table IV). Nearly every volume was half of what would have been used without the autostainer. The volume of each reagent used could be lowered further if a lower drop volume than 600 µL were chosen.

Table IV: Reagent Use Comparison for Each Sample

Reagent	Volume Specified in Original Protocol	Volume Used in Autostainer
Formalin	1.5 mL	1.5 mL
Phosphate Buffer Solution	12 mL	1.2 L (+ 7.2 mL)
10% Triton X-100	1.5 mL	0.8 mL
Bovine Serum Albumin	1.5 mL	0.8 mL
PECAM Primary	1.5 mL	0.8 mL
PECAM Secondary	1.5 mL	0.8 mL
BBI	1.5 mL	0.8 mL

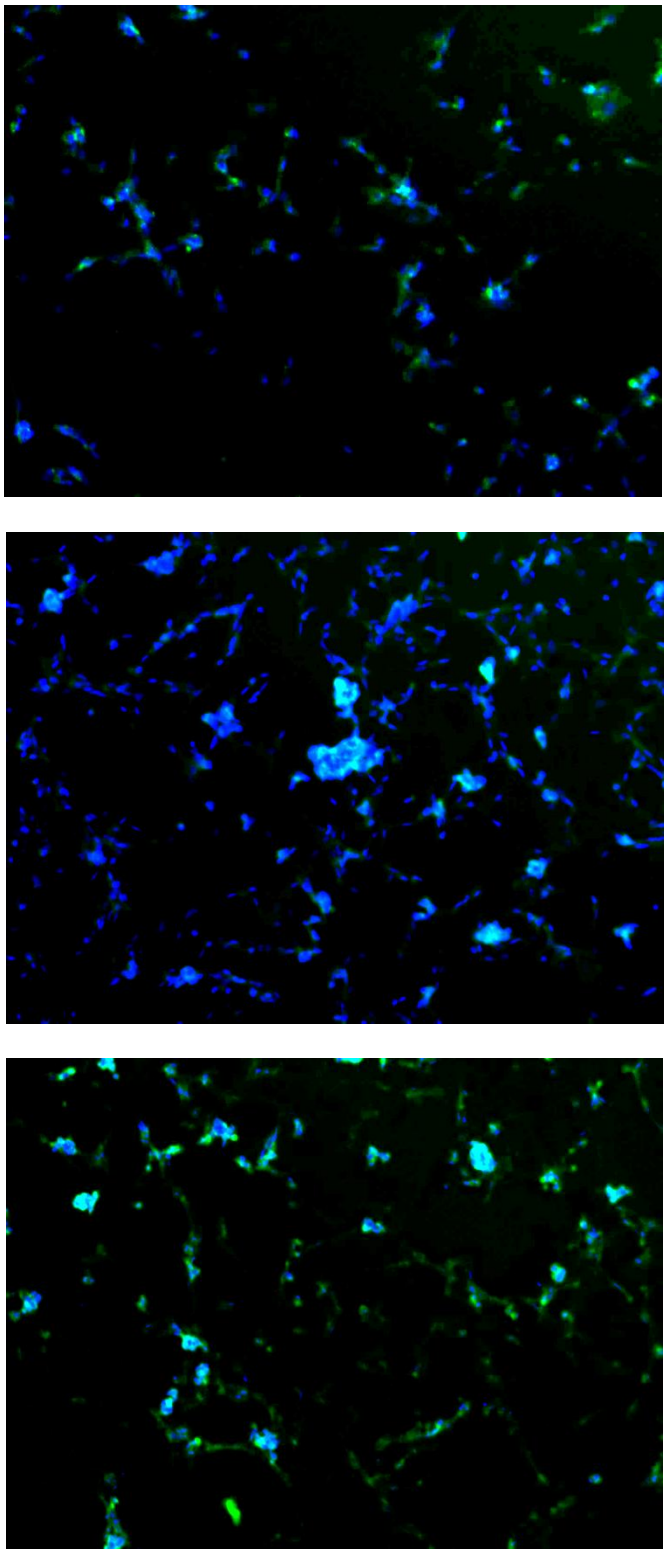


Figure 10: Snaps taken in ImageJ of the HUVEC sample.

Coverslip Adhesive Test

As seen in Table V the coverslip adhesive should be dried for at least 20 minutes before being placed in the autostainer (or exposed to any aqueous solution) to prevent sample movement or loss during the staining procedure. The autostainer uses a pump to forcefully blow air and buffer solution across each sample after each step to remove excess reagent from the slide. If the samples are not affixed to the slide correctly then they will either move away from the correct drop zone or be blown completely off the slide. The coverslip that was allowed to dry for 15 minutes was slightly wet before adhesive was added. The procedure used for the experiment is presented in Appendix C.

Table V: Adhesive Dry Time Results

Dry Time (min)	Coverslip Movement
0	Yes
5	No
10	No
15	Complete loss of slip
20	No
25	No

Discussion and Conclusion

Discussion

The Dako autostainer is now repaired and functional. The automated cleaning cycle proved that the autostainer could run with the newly replaced components. The PECAM and BBI staining was successful since both the PECAM green and BBI blue are distinguishable on the snaps in Figure 10, as expected for a HUVEC sample. The reagent volumes used in the automated HUVEC PECAM and BBI stain were also nearly half those used in dipping protocol. Although the autostainer uses significantly more phosphate buffer solution than the dip method the overall cost of the stain (especially if more than one slide was being stained) would still be lower. Phosphate buffer solution is relatively cheap compared to specialized antibodies used in immunohistology. Also, it is possible that successful staining can be achieved even with smaller drop volumes. In the HUVEC PECAM and BBI stain the largest reagent drop volume was used, 600 µL, but the machine can go as low as 100 µL.

Unfortunately the second test of the autostainer failed due to improper coverslip fixation to the slides. The researcher who performed the failed test hypothesized that the coverslips fell off the slides during the autostaining protocol either because the adhesive did not dry completely before buffer was added to prevent the cells from drying out. The coverslip adhesive test was performed to investigate proper coverslip mounting technique. The test showed that coverslips should be allowed to dry for about 20 minutes and that absolutely no liquid should be introduced to the adhesive until it has completed drying.

A guide was prepared to aid students in using the autostainer. Future work for this project will arise as students begin to use the autostainer and new problems arise. The first tests, however, should determine if smaller drop volumes can be used to successfully stain samples. Smaller drop volumes will allow for more samples to be run simultaneously and lower the volume of expensive reagents used in

the protocols. The guide can be modified and refined as students experiment and generate more data using the autostainer.

Conclusion

In conclusion, this project produced a functional autostainer that:

- reduces required reagent volumes in a staining protocol by nearly half and perhaps more
- reduces student exposure to dangerous reagents
- reduces the amount of time students spend performing staining protocols
- performs stains with consistent, repeatable conditions
- prevents user mistakes
- can stain more samples simultaneously than can be stained by hand
- reduces reagent and sample exposure to light

Future Work

Students need to begin to work with the autostainer to discover if there are more problems with it. The autostainer could be improved in a number of ways. The Dako software is not user friendly and not conducive to setting up new protocols. The software also does not allow for the stain to be paused after reagents have been added to the samples. If a protocol could pause after a certain step then the student could remove the samples for heating or other procedures that the autostainer cannot perform. Finally, a cooled reagent rack would keep staining reagents and antibodies more stable.

Appendices

Appendix A: Automated Cleaning Protocol

"Dako Autostainer Handbook Revision A", p 97

Section 17 | System Maintenance

It is recommended that the Dako Autostainer be cleaned after 150 slides have been processed on the instrument. The cleaning procedure can be divided into a manual phase and an automated phase.

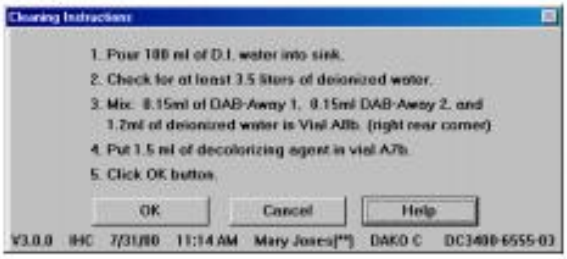
Manual cleaning consists of wiping down any areas exposed to reagents and buffer using deionized (DI) water and a detergent. Organic solutions are not recommended for cleaning. The slide racks should be removed after completion of the automated cleaning cycle and rinsed with deionized water.

The automated cleaning cycle entails running the cleaning protocol. The Dako Autostainer keeps track of the number of staining runs performed. The instrument displays a maintenance message listing the number of slides since the last cleaning cycle.

During the 20-minute, automated cleaning cycle the reagent probe will be incubated with cleaning solutions to remove any build-up of chromogen. In addition, the wash head dispenses DI water on each of the 48 slide positions to remove any buffer deposits or reagent residue remaining after the staining run.

Automated Cleaning Cycle

1. Select the **CLEAN** button on the ***OPTIONS*** screen. The ***MAINTAINENCE*** screen displays the number of slides completed since the last cleaning cycle.
2. Select the **CLEAN** button on the ***MAINTAINENCE*** screen. The **Cleaning Instructions** window appears with instructions on how to prepare the instrument for a maintenance run.



NOTE: The following reagents are required to perform a cleaning run:

- DI Water
- DAB-Away 1 solution
- DAB-Away 2 solution
- Decolorizing Agent solution

3. Prepare the cleaning reagents as instructed in the ***CLEANING INSTRUCTIONS*** window and load into the indicated rack positions. (Positions "A8b" and "A7b" refer to positions A8 and A7 in the back rack.) Pour 100 mL of deionized water into sink and provide 3.5 L deionized water in a water reservoir *(The "01" hardware version of the Dako Autostainer does not have a reservoir for deionized water)*. Select the **OK** button. The Autostainer starts the cleaning run and the Run Log is displayed. When the run is completed the ***MAINTENANCE*** screen appears. Select **OK** to return to the ***OPTIONS*** screen.

   NOTE: Do not use bleach in the Dako Autostainer. Bleach may react with other chemicals and create toxic fumes.

Appendix B: PECAM & BBI Staining Protocol

Original PECAM & BBI Stain by Elizabeth Curiel:

1. Remove cultured medium & fix cells with 10% formalin for 15 min. Use enough to cover the slip.

- I used 1.5 mL per well.

2. 3 PBS (phosphate buffer saline) washes 5 min ea. Use enough PBS to cover slip.

- I used 1 mL.

3. Permeabilize the cells in 0.2% Triton X-100 in PBS in order to get 0.2%. Incubate for 30 min.

- 1.5 mL per well.

4. 3 PBS washes 5 min. ea. Use enough PBS to cover slip.

- 1 mL per well.

5. Block for non-specific antibody in 6% BSA (bovine serum albumin) for 50 min.

- 1.5 mL per well.

6. Incubate in primary antibody and 6% BSA for 50 min. (antibody concentration between 0.5-10µg/mL).

- 1.5 mL per well.

7. 3 PBS washes 5 min. ea. Use enough PBS to cover slip.

From this point on less light the better (light sensitive).

8. Incubate with secondary antibody in 6% BSA for 50 min. (antibody concentration 5µg/mL).

- 1.5 mL per well.

9. 3 PBS washes 5 min. ea. Use enough PBS to cover slip.

10. Counter stain BBI for 15 min.

- Always keep wrapped in foil. Mix 10 µL stock in 10 mL of Milli Q-Water (100:1).

11. 1 PBS wash for 5 min.

- 1 mL per well.

12. Mount ProLong Gold for Fluorescent Secondaries onto two wells. Enough to cover slip.

This protocol was modified to be used with the autostainer. The exact protocol of the stain performed on 2/4/12 is described below:

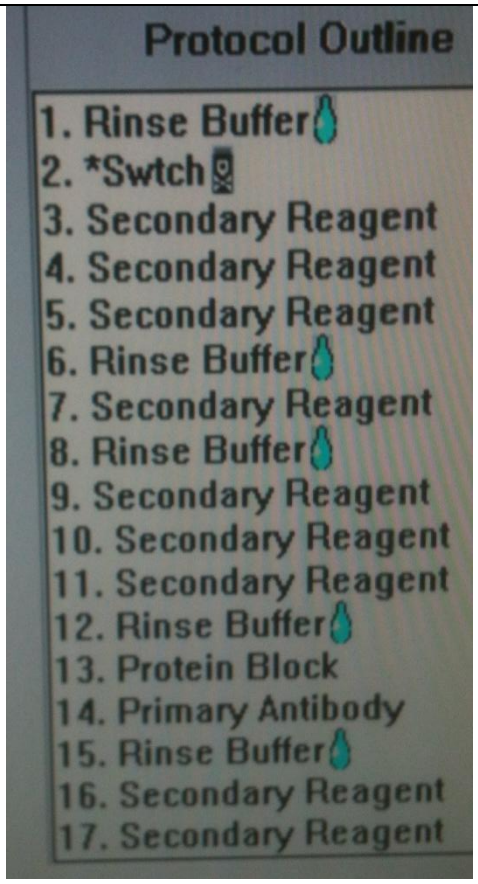
Table BI: Materials List for PECAM & BBI Stain of HUVEC as Performed on 2/4/12

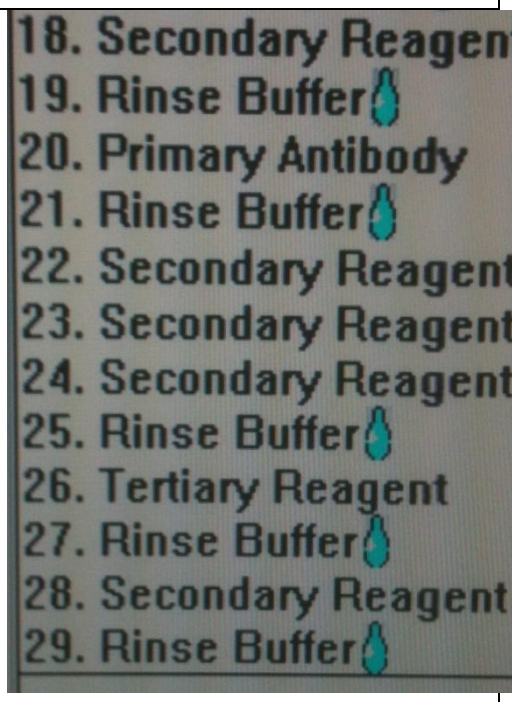
Material	Amount
DI Water	2 L
PBS Packets	2
PECAM-1 Primary 2mg/mL	2 µL
PECAM-1 Secondary 0.5 mg/mL	8 µL
BBI	0.8 µL
Milli Q-Water	0.8 mL
1% Triton X-100	0.16 mL
BSA	2.4 mL
Formalin	1.5 mL
HUVEC Sample	1 Sample

Note: I tried to get proper screen shots from the computer but it does not have access to the internet, USB, or a CD burner. The only option was phone pictures of the screen.

1. The protocol steps listed above were programmed into the autostainer (Table BII) and the incubation and reagent types were entered.

Table BII: Steps of Protocol as Entered in the Autostainer





All of the waste from the protocol was collected in the hazardous waste bin (as seen in step 2 in Table BII). PBS buffer washes had to be performed as 'secondary reagent' steps since the autostainer's standard 'rinse buffer' step does not incubate for 5 minutes. Every step in the protocol used 600 µL of reagent, the maximum allowable amount.

2. Reagents were prepared to the correct concentrations based on their stock.

3. Pipette a sample of tenth passage HUVEC tissue sample onto a glass slide. Pipette 1.5 ml of 10% formalin over the cells and incubate 15 minutes until samples are fixed.

4. The protocol was started on the computer and the sample was placed into the autostainer as outlined by the slide map screen (Figure B1). The drop area was set to the middle of the slide as that is where the sample was mounted.

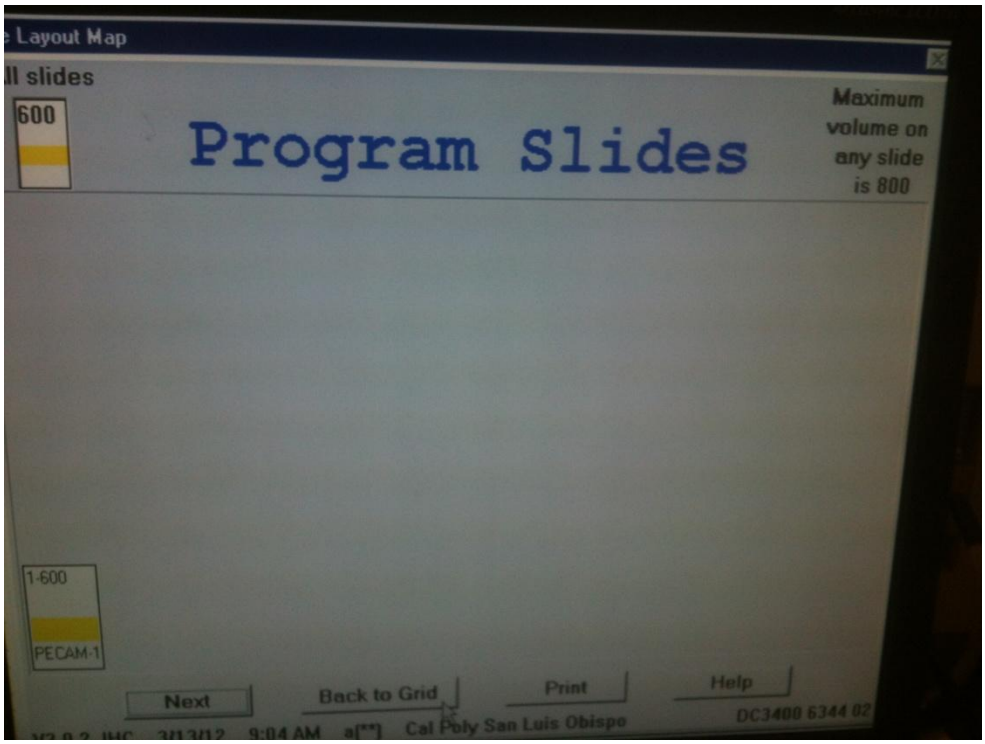


Figure B1: The slide map screen displaying the position of the only slide of the experiment. Note in the top left that the volume is set to 600 µL and that the middle section of the slide is highlighted.

5. The autostainer was wrapped in tin foil to minimize light exposure to the light sensitive antibodies (Figure B2).

Figure B2: The autostainer was wrapped in tin foil.

6. Next, reagents were added as outlined by the reagent loading screen (Figure B3).

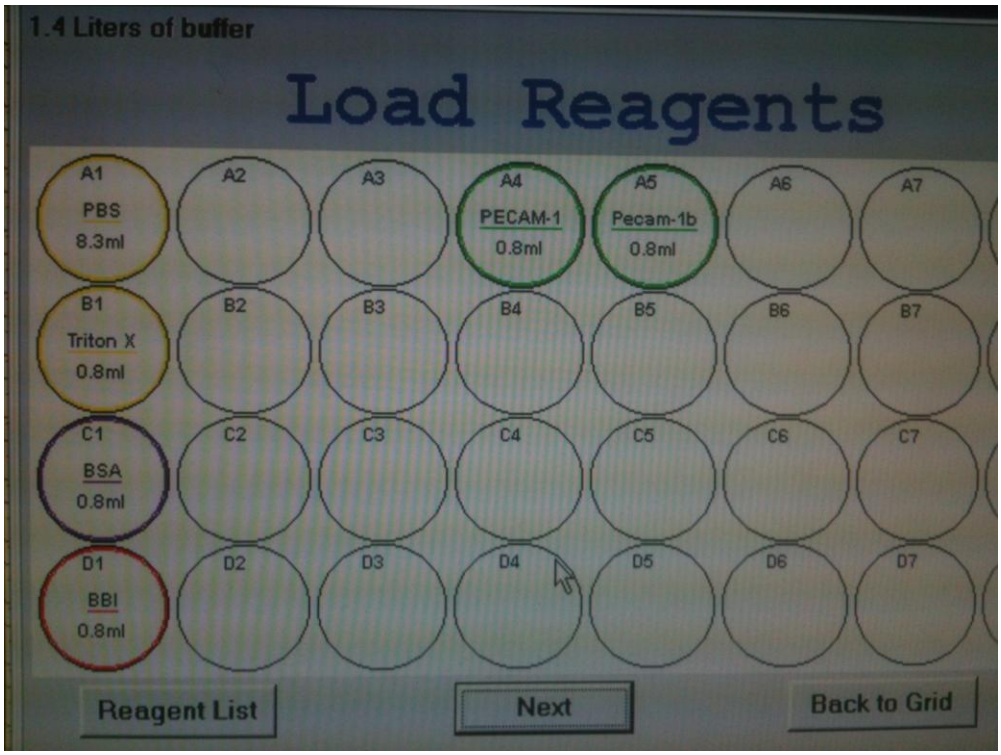


Figure B3: Reagent loading screen. Only 0.8 mL of each antibody was used.

7. The DI water and PBS buffer carboys were filled to the amount specified by the autostainer (Figure B4). 2.0 L of PBS buffer were made by mixing two packets of XXXX PBS mix into 2.0 L of DI water. No DI water was necessary for this protocol.

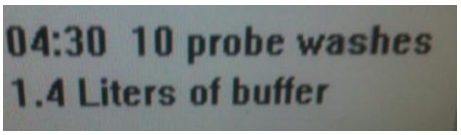


Figure B4: Estimated time and buffer usage for this protocol. Not all of the PBS was used.

8. The procedure was started and took approximately 270 minutes. The sample was removed in darkness, transported to another room, and imaged.

9. Excess reagents and the waste carboys were disposed of. Excess PBS was saved for the next run.

Appendix C: Coverslip Adhesive Drying Test

1. For each slide the same mounting procedure was used but the drying time was changed. A single bead of toluene mounting adhesive was placed on the center of a glass slide and, using tweezers, a single circular cover slip was placed onto the bead. Capillary action distributed the adhesive across the area of the slip. No pressure was applied to the coverslip.

2. Coverslips were allowed to dry for 25, 20, 15, 10, 5, and 0 minutes before initiating the stain protocol. The protocol was the same as presented in Appendix B but with all of the reagents replaced with PBS.

3. The protocol was initiated and let run. After complete the samples were checked for movement or removal of the coverslip.

Appendix D: Autostainer Protocol for Students at Cal Poly

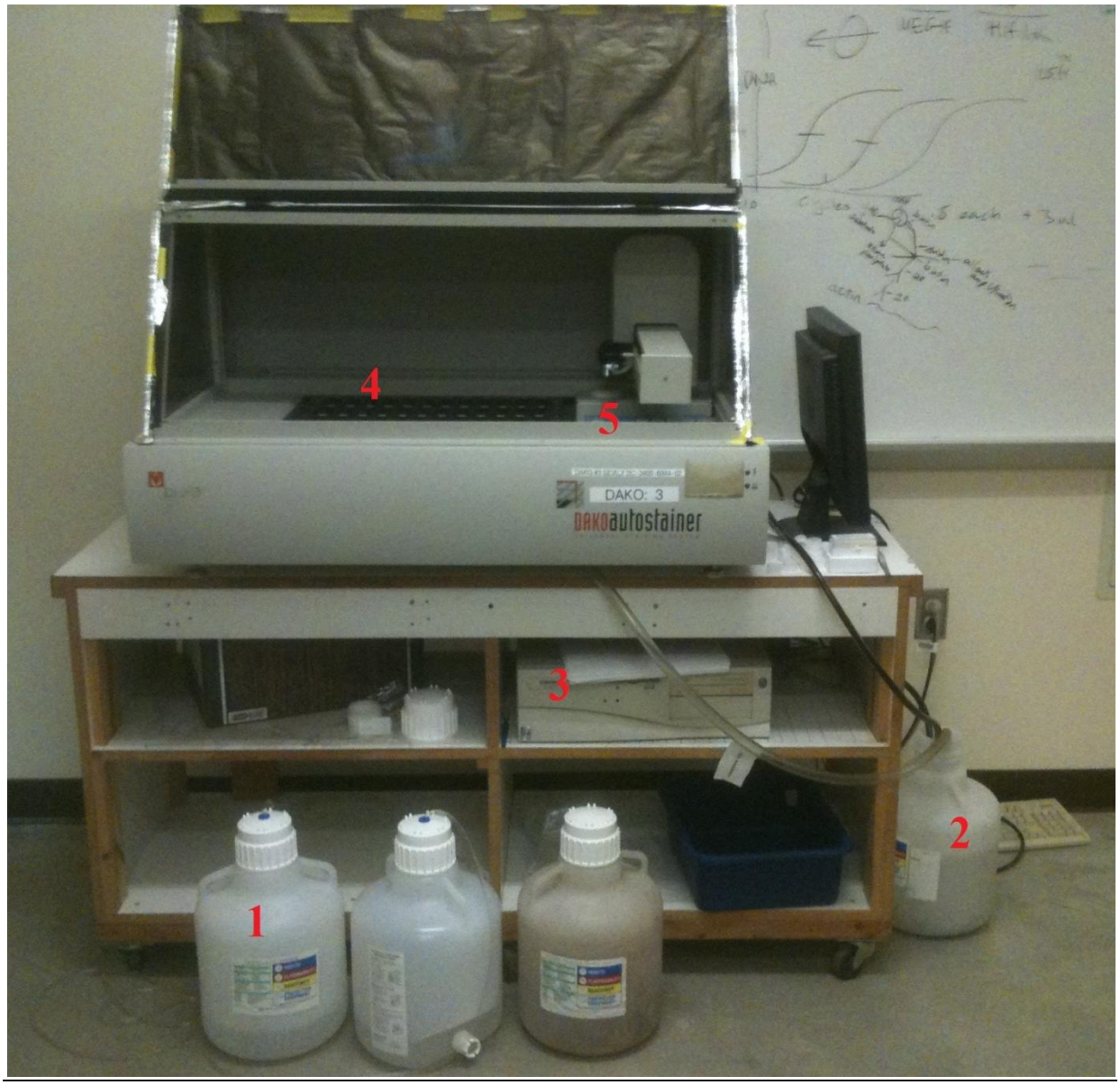


Figure D1: The autostainer with all components. 1 is the DI water and buffer carboys, 2 is the waste carboy, 3 is the PC, 4 is the slide rack, and 5 is the reagent rack.

Programming a Protocol

1. Make sure autostainer is plugged in and turn on the computer. After it starts up open the Autostainer software. Login.

Username: a
Password: a

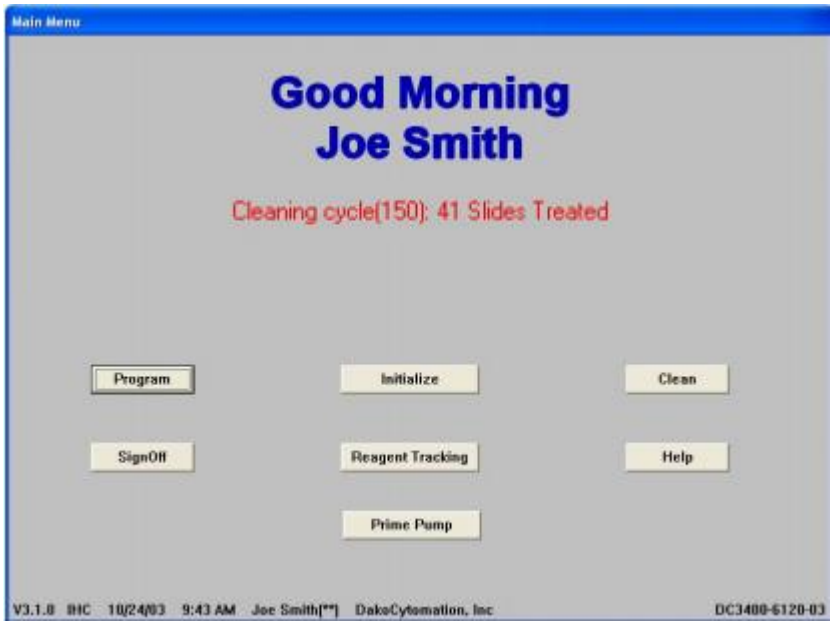


Figure D2: Main Menu (Dako Autostainer Handbook, 7).

2. Click 'Program'. You should now be on the Programming Grid page.

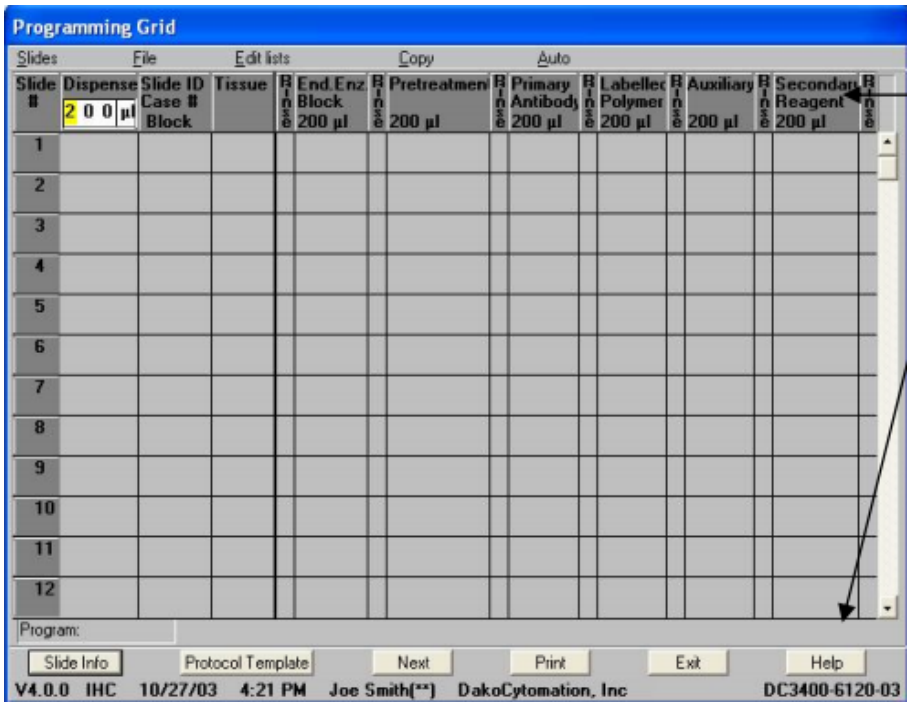


Figure D3: Programming Grid page (Dako Autostainer Handbook, 12).

3. Click ‘Protocol Template’. You should now be on the Protocol Template Design page.

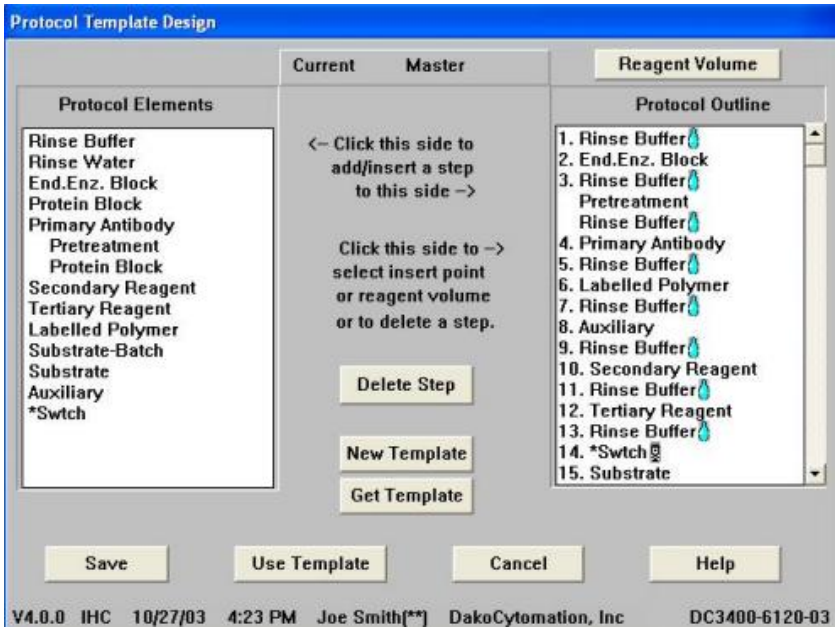


Figure D4: Protocol Template Design page (Dako Autostainer Handbook, 15).

4. Arrange the general order of your protocol on the right box using the options on the right box.

    - The first step should be ‘*Switch’ to ensure that all waste goes to the hazardous waste carboy.

    - PBS washes can be programmed with the ‘Rinse Buffer’ action.

    - Each step should be followed with a ‘Rinse Buffer’ step to clear the reagent off your samples.

    - Fixing steps should be performed before samples are brought to the autostainer.

5. Click ‘Reagent Volume’ on the top right of the screen and select your desired dispense volume.

6. Click ‘Save’ and name it something meaningful so you do not lose it.

7. Click ‘Use Template’. You should now be back on the Programming Grid page.

8. Click ‘Slides’ on the top left and set how many slides you want to stain.

9. Set up reagents and incubation times. Click ‘Edit Lists’ and select the appropriate reagent category.

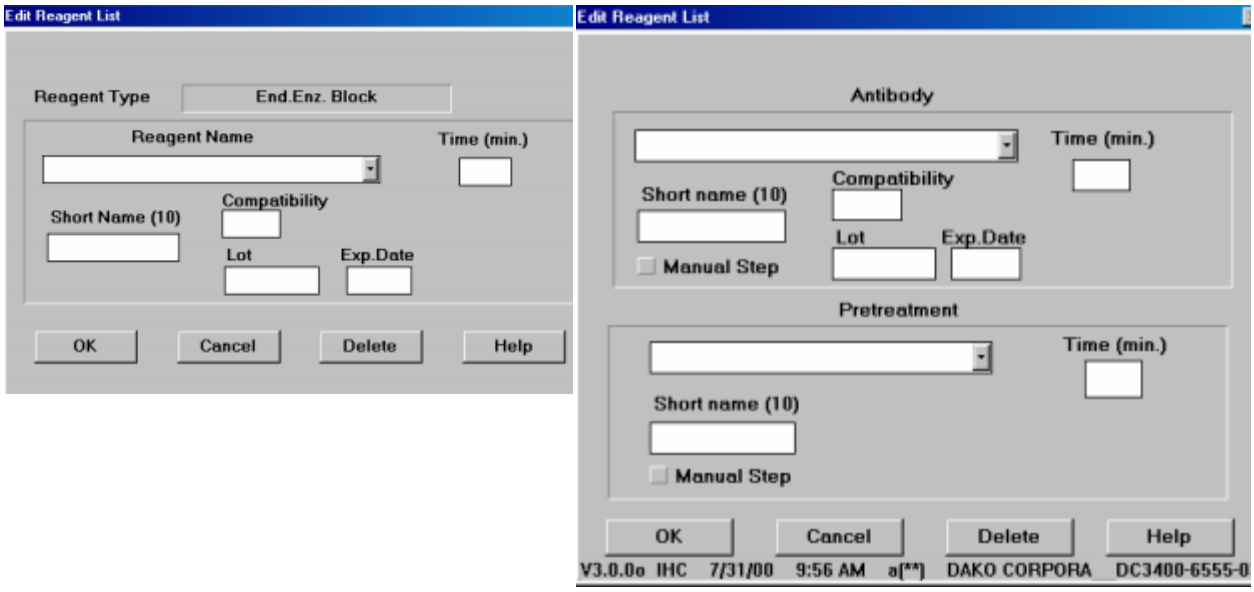


Figure D5: Reagent addition menu for regants and antibodies (Dako Autostainer User, 69 and 71).

10. Fill out the reagent name, abbreviated name, and incubation time. Hit enter.

11. Repeat steps 9 and 10 until all reagents and antibodies are defined.

12. To set the correct reagent to each step of the protocol, click on the gray box of the desired step on the first slide. A list of available reagents will appear. Select your desired reagent. The software will prompt you if you want all slides to have the same reagent. Click yes/no. Note: incubation time is associated with the incubation time as defined in step 10, it must be changed in the 'Edit Lists' menus, not on the Programming Grid.

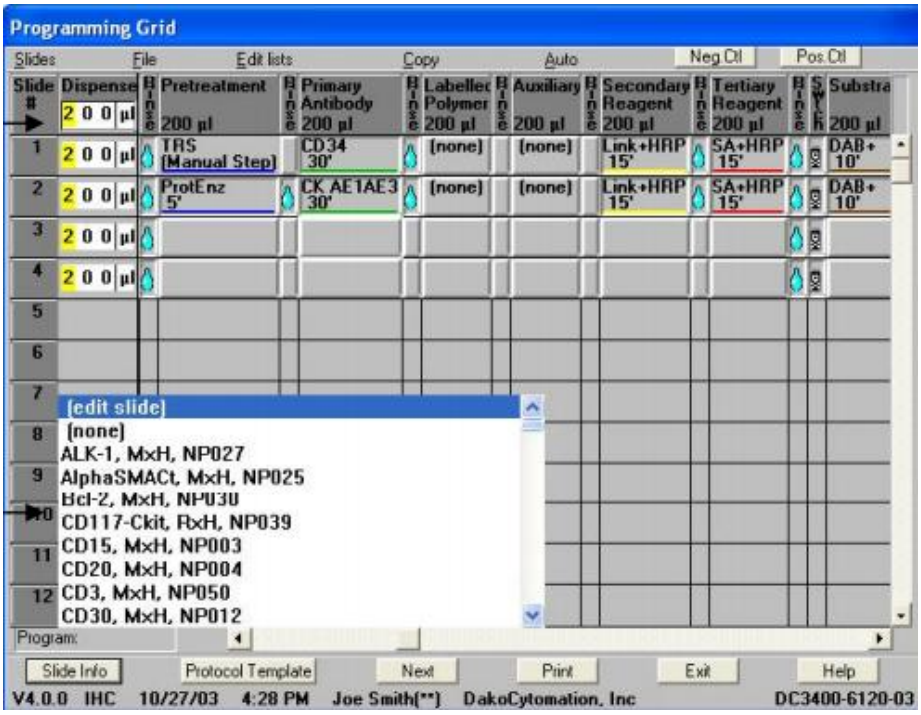


Figure D6: Setting reagents to each step of protocol (Dako Autostainer Handbook, 19).

13. Repeat step 12 for each reagent on each slide.

14. Save your protocol. Click 'Next' on the bottom of the Programming Grid page. Note the run time, PBS required, and DI water required.

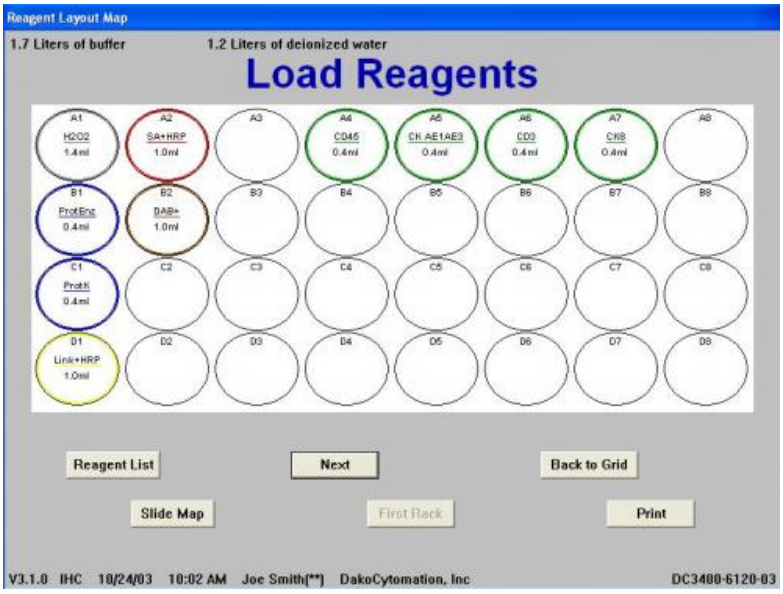


Figure D7: Reagent Layout Map (Dako Autostainer Handbook, 31).

15. Take a picture or write down the order and volumes of the reagents as they are displayed on the Reagent Layout Map.

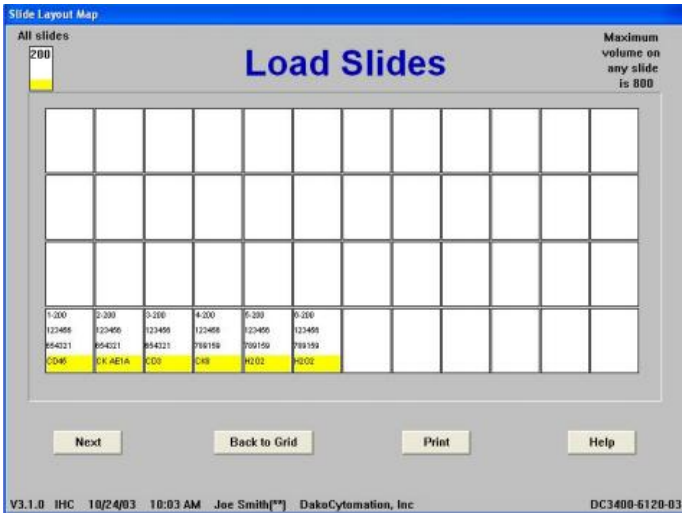


Figure D8: Slide Layout Map (Dako Autostainer Handbook, 33).

16. Click 'Next'. Take a picture or write down the order of the slides as they are displayed on the Slide Layout Map.

17. On the top left of the Slide Layout Map the reagent drop position of for all of the slides can be selected. The drop position can be changed on individual slides as well by clicking on the bottom, middle, or top of the slide. A yellow bar indicates where the reagent will be dropped onto the slide.

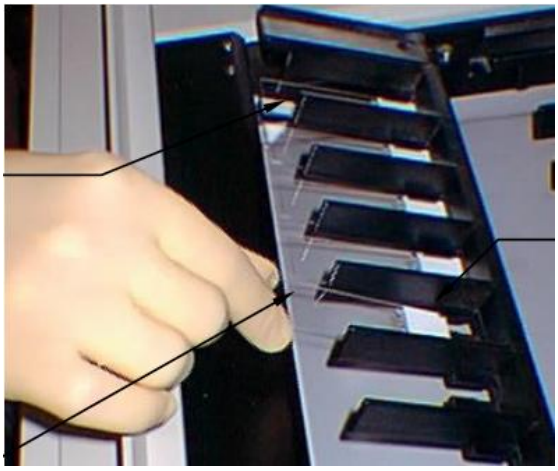


Figure D9: Slide loading orientation (Dako Autostainer User, 83).

18. Programming is now complete. Either save and shutdown the computer or prepare reagents and samples.

Sample and Reagent Preparation

19. Bring the information from steps 14, 15, and 16 above along with the reagent racks and carboys to wherever you will be preparing the reagents and samples.

20. Prepare tissue samples how you normally would. Perform fixing procedure. Attach tissue to coverslips. Mount coverslip to slide at location where you set the drop location to in step 17, above. Make sure the bottom of the coverslip and top of the glass slide are completely dry before mounting. Allow at least 20 minutes for the mounting adhesive to dry. Label the slides so you know which position to put the sample in inside the autostainer (from step 16). After 20 minutes a small amount of PBS should be placed onto the samples to prevent drying out. Keep the samples wet until the autostainer protocol is started.

21. Make the required amount of PBS as determined in step 14 and put in the buffer carboy. Fill the DI water carboy with the required amount of DI water as determined in step 14. Prepare the reagents and antibodies and put them in the reagent rack in the correct positions (from step 15). Reduce exposure to air and light using the tube caps and tin foil if necessary. Use the following equation for diluting stock solutions to the required concentrations.

$$C_1V_1 = C_2V_2$$

Starting the Staining Run

22. Bring carboys, reagents, and tissue samples to the autostainer. Turn on the computer, open up your saved protocol, and hit 'Next'. Load the reagents as outlined by the Reagent Layout Map and place into the correct block in the autostainer. Click 'Next'. Load the slides as outlined by the Slide Layout Map in

the orientation outlined in Figure C8. Put the slide racks into the autostainer. Put the caps back onto the buffer and DI water carboys, making sure they are attached to the correct inputs. Click 'Next'.

23. Make sure all of the waste tubes are firmly placed into waste carboys. Make sure that the total volume used in your protocol will not overflow the carboy!

24. Click the 'Prime Pump (Buffer)' button and wait until the autostainer completes the priming procedure. Repeat until the buffer flows out of the autostainer dispenser with no air bubbles. This may several tries. Make sure the tubing is not pinched and that the intakes are immersed in the buffer.

25. Repeat step 23 but with the 'Prime Pump (Water)' button (only if your protocol uses DI water).

26. Remove the caps and tin foil from the reagent tubes (turn off lights if you have to), close the lid of the autostainer, and click 'Start Run'.

27. After the completion of the stain remove your samples and image them at the appropriate wavelengths. Again, avoid light exposure.

28. Pour some DI water into the autostainer sink, clean the metal mesh sink guard if necessary. Clean all reagent tubes, empty the waste carboys, and shut off the computer. Dispose of your samples appropriately.

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