

Optimized Immunohistochemistry
Workflow Facilitated by
New Dako Autostainer Link 48 Software

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Abstract

Automated immunohistochemistry systems, including instruments and reagents, are used by many histopathology laboratories. Dako currently supplies one such instrument, the Autostainer Link 48. This has a capacity of 48 slides per batch/run and uses a pipetting arm to aspirate and dispense reagents. Until recently, the production software (version 3.0) resulted in staining times of up to 4.5 hours, excluding antigen retrieval and ancillary steps. However, a new software version (3.1.1) has just been introduced, and was evaluated in the authors' laboratory. Using software version 3.1.1, to stain over 40 slides, resulted in run times of about 2.5 hours compared with over 4.0 hours using software version 3.0 software. The new software version also reduced buffer usage and generated less waste than the previous version. Including dewaxing, antigen retrieval (in Dako's PT Link), staining on the Autostainer Link 48, counterstaining, dehydration, clearing and coverslipping, the Dako system processes 96 slides in approximately 6.5 hours and 144 slides in about 9.0 hours, which is greater throughput than competitive platforms. Moreover, the new software version significantly reduces hands-on time per slide compared to other instruments.

Introduction:

Immunohistochemistry (IHC) is a key diagnostic tool in tissue pathology (1). The number of antibodies available for application to routine formalin-fixed, paraffin-embedded (FFPE) tissue sections is increasing rapidly. While some laboratories still employ manual methods to perform immunohistochemical stains, many institutions increasingly use automated staining platforms. Some of these platforms are "open" and can employ any available primary antibody or detection system; and others are "closed", requiring at least the use of detection systems provided by the instrument manufacturer.

Over the years, automated staining instruments have made use of various mechanisms to dispense immunohistochemical reagents. For example, "capillary gap" technology was used to draw reagent between two directly opposed slides (e.g., patient and control) (2). However, most systems have relied upon robotic pipetting mechanisms, sometimes with ancillary delivery systems for wash buffers and other bulk fluids. Accordingly, the time taken for any IHC instrument to perform a full staining run depends not only on incubation times of individual reagents, but also on the time it takes for robotic arms to move from one slide station to another, or from reagent wells to slides. Hence, the actual throughput rate for any automated IHC instrument is based on both the total time required for the instrument to perform a full staining run and on the numbers of slides stained.

The capacity of currently available IHC instruments varies from about 30 to 60 slides. The most commonly used systems are from Ventana, Leica and Dako (3-5), all of which provide dedicated reagent sets for use with their instrument platforms. While other manufacturers also supply instrument and reagent packages, most of these function similar to the Dako system (3). With previous versions of the operating software, the Dako Autostainer took longer to complete a full staining run, not including antigen retrieval in the PT Link instrument (6). Even though the Dako system could stain 48 slides at once, compared with 30 for Ventana and Leica, it was generally considered to have a lower throughput, which would be a disadvantage for a busy diagnostic laboratory.

Until recently, the Dako system's longer staining times have been due to software scheduling issues for the pipetting arm – not to the staining method *per se*. To address this issue and improve run times, Dako has developed new software (version 3.1.1). In the current study, the authors compared the processing capacity and other features of the version 3.1.1 software running on an Autostainer Link 48 instrument, in parallel with an Autostainer Link 48 instrument running the version 3.0 software. The study was conducted in the Gamma-Dyncare Molecular and Cellular Pathology Laboratory in Bowmanville, Ontario, Canada.

Results

Comparison of software 3.0 and 3.1.1

The new software version (version 3.1.1) for the Autostainer Link 48 instrument resulted in 30% faster run times (up to two hours time saved) compared with version

3.0 with full batches of 48 slides (Figure 1). Predicted run times were also improved.

Importantly, faster run times did not affect overall IHC staining quality – at least not for the 39 primary antibodies

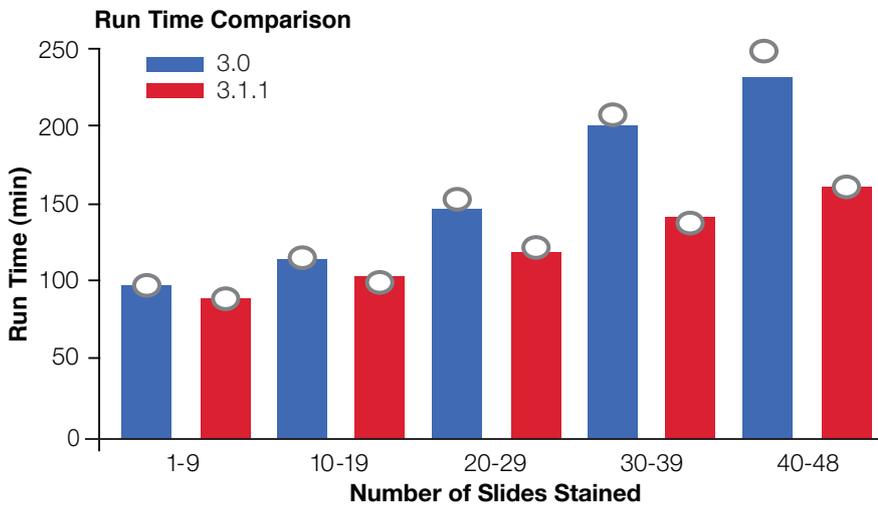
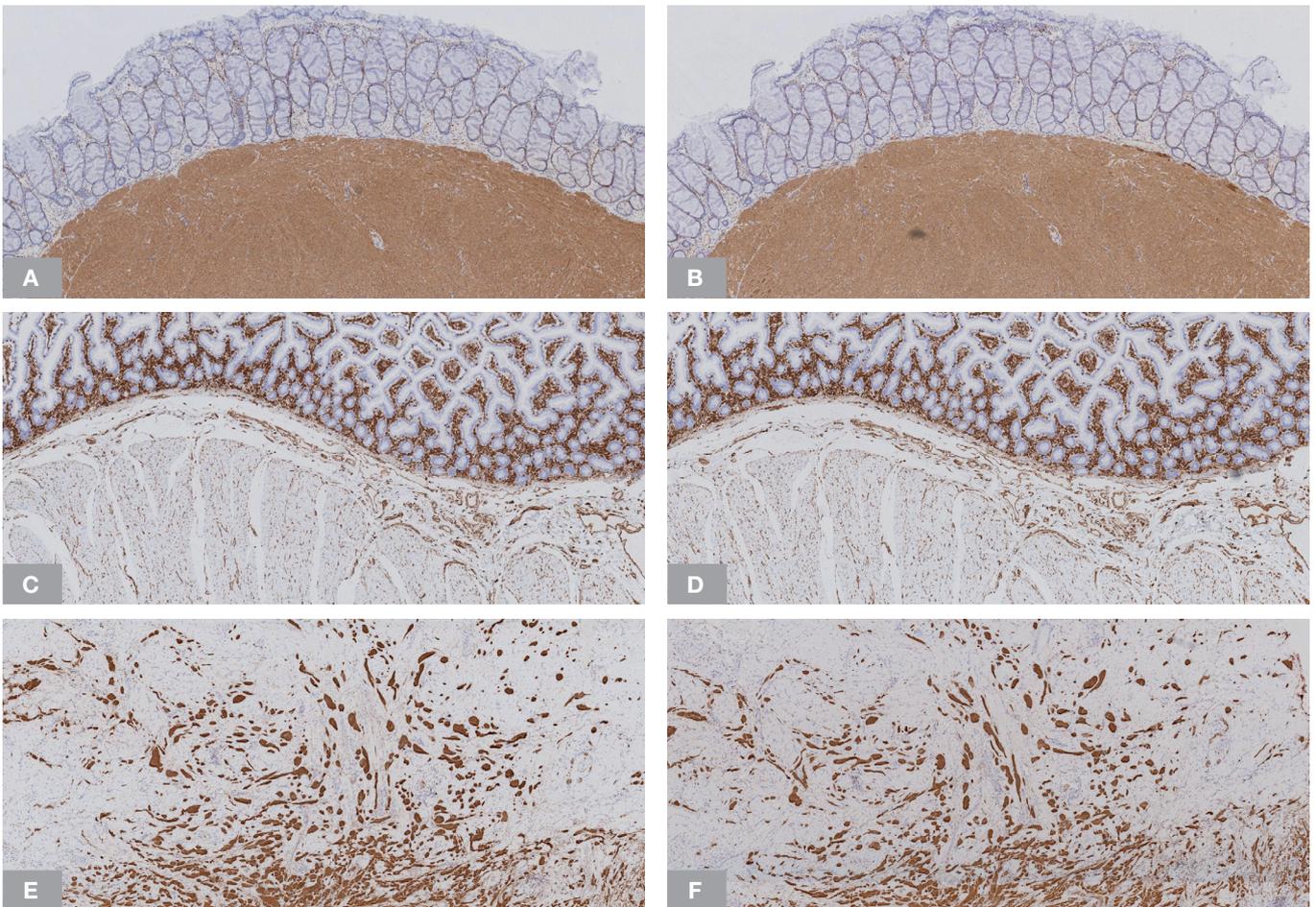


Figure 1: Run time comparisons for a range of slide batches

Red bars indicate version 3.1.1 software; blue bars indicate version 3.0 software. The light blue circles above each bar represent the mean predicted run time for each group (batch size as indicated on the x-axis; n = 4 or 5 per group). The maximum variation was seen in the group of 30-39 slides stained using version 3.0 software (mean \pm S.E. (n): 200.3 ± 16.61 (4)). Run time is clearly improved with the new software, especially with larger slide loads.

Figure 2: Examples of results from pairs of slides stained using both versions of software.

Examples of results from pairs of slides stained with versions 3.1.1 (A, C, and E) and 3.0 (B, D, and F) software. Equivalent results were consistently attained. A & B: submucosal nodule stained for actin; C & D: normal small bowel stained for vimentin; and E & F: granular cell tumour stained with S100. IHC was performed on formalin-fixed, paraffin-embedded (FFPE) sections using Dako FLEX Ready-to-Use Primary Antibodies and the EnVision™ FLEX detection system.



and over 1,000 slides evaluated in this study. There was, however, some indication that staining was slightly stronger using version 3.1.1 software, with no increases in background staining. Examples of the paired staining results are shown in Figure 2. In no instance was the difference in staining intensity greater than 0.5 (on an arbitrary scale 0 to 3+). More importantly, any differences in staining intensity would not affect the interpretation of the stain.

Consistency of Staining

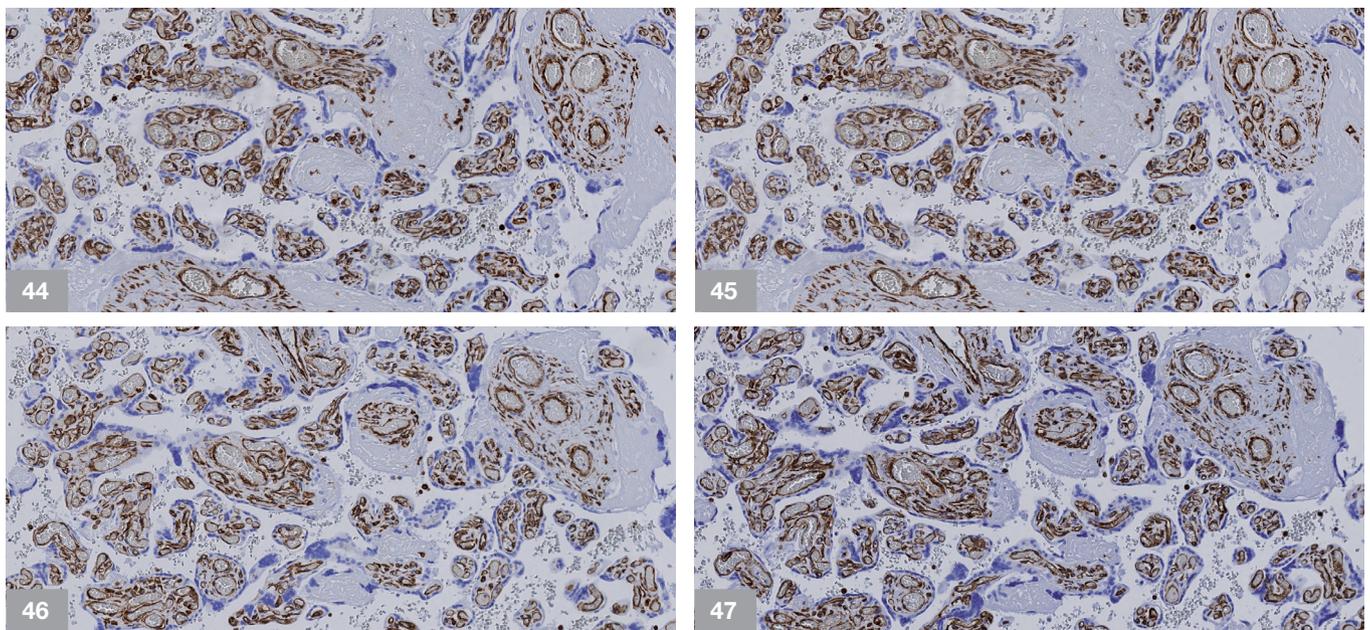
Consistency of staining using version 3.1.1 software was very good. Sample microscopic views of slides from the second of four full runs are shown in Figure 3. The quality of staining is clearly excellent, and is equivalent to results using version 3.0 software. The consistency is at least as good as results seen with competitive

instruments from Ventana and Leica, recently evaluated in the authors' laboratory (unpublished data).

A few slides did exhibit staining variability. However, this was in tissue located very close to the label end of the slide. Other minor staining defects were rarely observed in these slides or in the paired sections stained with other antibodies. These appeared to be either roughly circular or angular, with reduced staining, and are likely due to small bubbles in one or more reagents. The reader should be aware that such artifacts are not restricted to the Dako Autostainer platform; these have been seen in all other systems tested in the authors' laboratory over the past few years. In some cases the other instruments exhibited significantly more of these artifacts.

Figure 3: Example of consistency of staining using new software.

Example of consistent staining, revealing equivalent results in four slides stained on adjacent stations (44 to 47) on an Autostainer Link 48 running version 3.1.1 software. FFPE sections of placenta were stained for vimentin using the Dako FLEX Ready-to-Use primary antibody and the EnVision™ FLEX detection system. Similar results were noted in all experiments, with only minor differences in staining quality.



Additional Studies

■ Reduced scanning time

The formal study of scan times confirmed initial observations: scan times are shorter using version 3.1.1 software than with version 3.0.

As might be expected, scan times were longer when more slides were introduced using version 3.0 software. However, a very interesting finding was that the scan time with version 3.1.1 software was relatively consistent regardless of the number of slides stained:

approximately two minutes per run. This is shorter than the minimum scan time using version 3.0 software with only a few slides on the instrument (approximately three minutes), and much shorter than the *circa* six minutes required for a full batch. Although this results only in a four-minute saving, there is an added benefit in those instances in which low or expired reagents are detected during the scan, necessitating a second scan after adding or replenishing those solutions.

Lower buffer usage and less waste

With the Autostainer Link 48 instrument running near full capacity on version 3.1.1, approximately 25% less buffer for slide washing was used compared with version 3.0 as shown in Figure 4A.

Small numbers of slides resulted in lower buffer savings, and there was little difference with 10 or fewer slides (Figure 4A). Interestingly, the predicted volume of buffer used was more accurate using version 3.0 than version 3.1.1, although the latter actually used considerably less buffer.

The new software clearly resulted in less waste than the old version in both full runs of 48 slides. This applied for both hazardous and non-hazardous waste (Figure 4B).

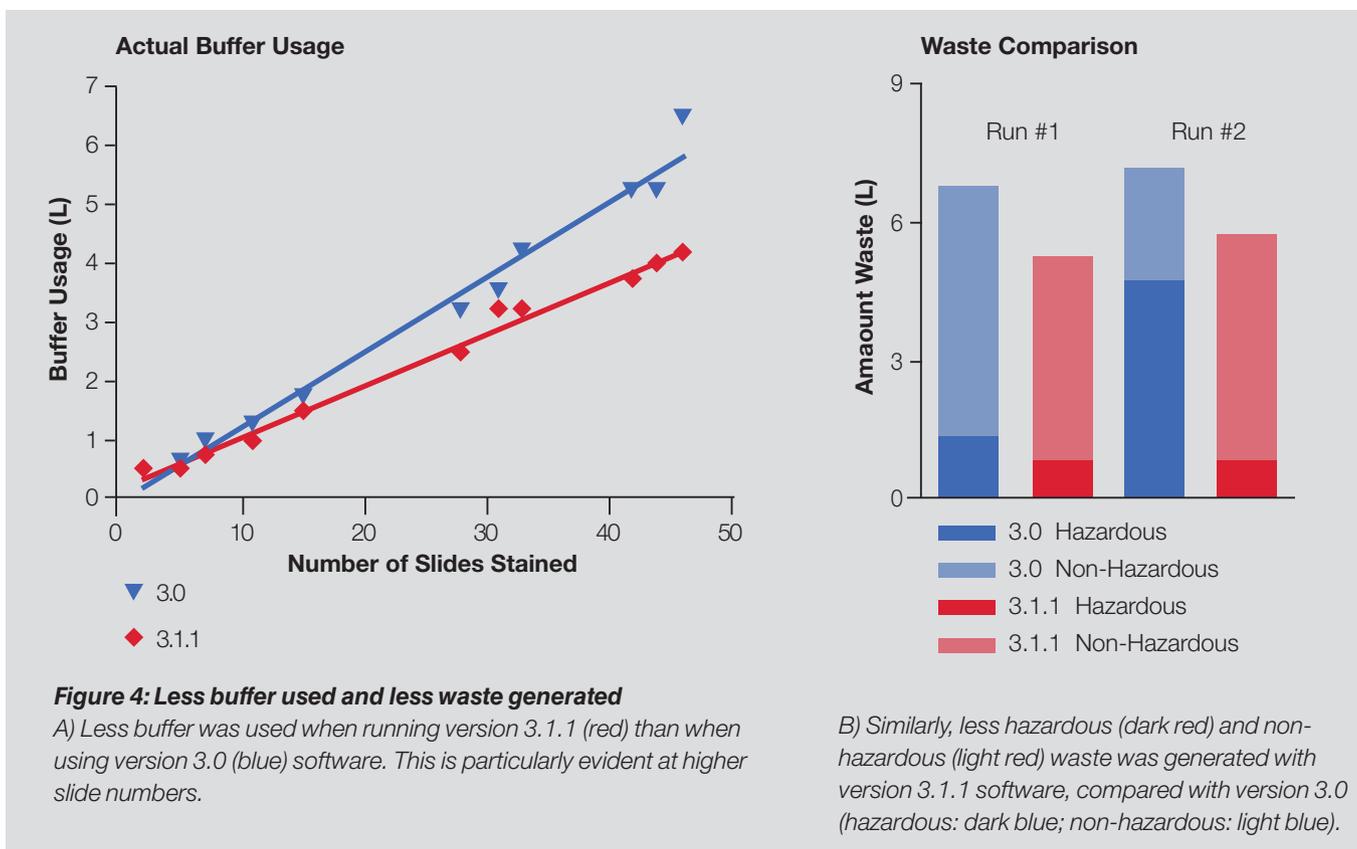
When using instruments with the new software, these results indicate that bulk solutions will need replenishing less frequently, and that exhaust will not need to be disposed of as frequently.

Discussion

The key question to be addressed during the experiments reported herein was if the new software improves slide throughput. The data in Figure 1 clearly show a noteworthy reduction in run time, especially with full or relatively full slide batches.

However, this does not reflect the total staining time, since there is preparatory work, antigen retrieval in the PT Link instrument, and finalizing the stains, including dehydration, clearing and coverslipping, all of which add to the total process time. This is depicted graphically in Figure 5 for Dako instruments using both versions of the software. Clearly, the new software allows the operator to perform three full runs of stains in less time than it took to perform two runs with the previous software version. Two runs of slides (96 in total) can be completed within 6 hours and 30 minutes; and three runs of slides (144 in total) can be done in just 9 hours. Thus, in a typical histology laboratory with staggered shifts, a single instrument could produce almost 150 slides per day using the new software (not including over-night runs).

Figure 5 also illustrates the workflow for the Ventana Ultra and Leica Bond III compared to Dako instruments running both software versions. Both the Ventana and the Leica systems have capacities of 30 slides compared to Dako's 48. Staining times for the Ventana and Leica systems are based on product claims (4;5) or earlier studies in the authors' laboratory (unpublished). Examination of Figure 5 shows that neither the Ventana nor the Leica instrument has the same throughput as the Dako platform running the new software version. The Ventana and Leica instrument take longer to process 90 slides in three runs than the Dako platform takes to stain 96 slides in two runs.



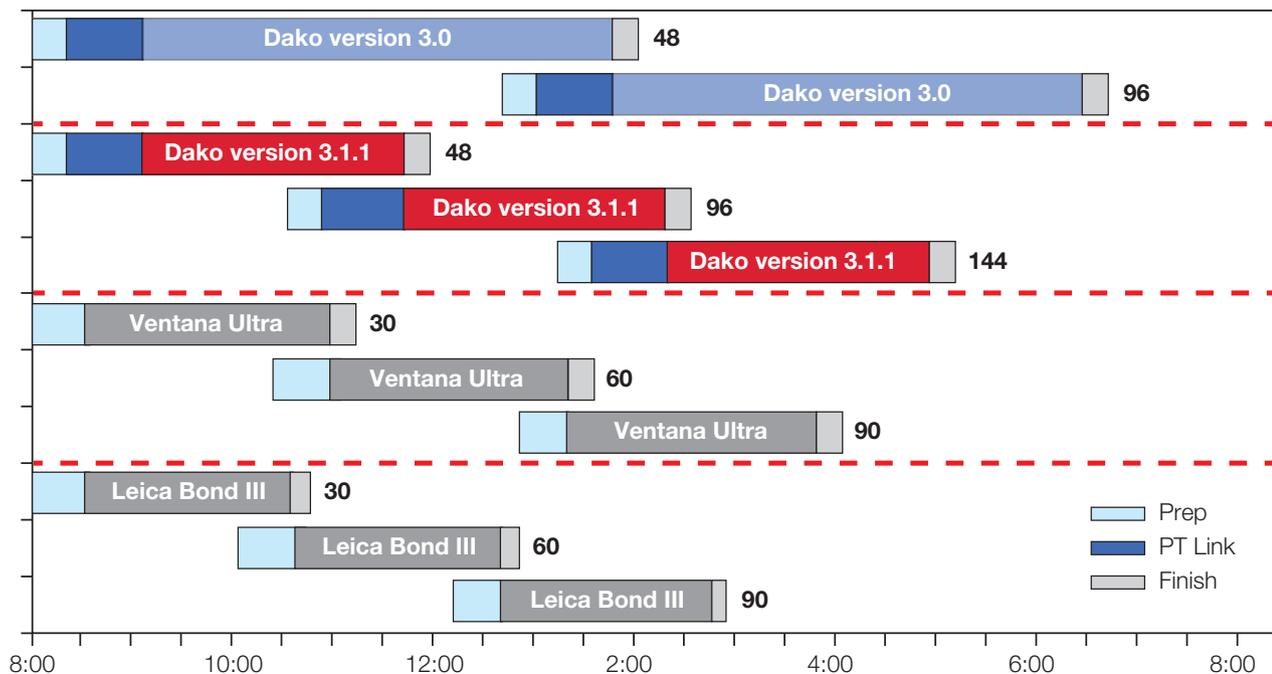


Figure 5: Illustration of approximate throughput of Autostainer Link 48 instruments with old and new software

Illustration of the approximate throughput of IHC stains using Autostainer Link 48 instruments loaded with version 3.1.1 (red) or version 3.0 (blue) software. Clearly, the newer software version can complete three runs in less time than the older version can complete two runs. One hundred and forty-four (144) slides can be stained in nine hours, including preparatory time, antigen retrieval in PT Link instruments and the ancillary steps needed to coverslip slides. For comparison, estimated throughput for Ventana Ultra and Leica Bond III instruments (based on the manufacturers' published data and personal observations) are included. Note that the new Dako software allows 96 slides to be stained on the Autostainer Link 48 before either of the competitors illustrated can complete 90 stains.

One perceived disadvantage of the Dako staining system is that slides have to be transferred from the antigen retrieval solution in the PT Link module to the Autostainer Link 48 instrument. This hands-on step is, however, a very short one. We measured the operator input needed to run the Dako staining system and found that this ranged between 40 and 50 minutes for a run of 48 slides. Earlier studies of the Ventana and Leica systems by the authors found that the hands-on time was in the order of 40 to 45 minutes per run. Thus, the Dako system appears to require more manual effort. However, when taking into account the number of slides stained in a full run on all three instruments, the actual hands-on time per slide for the Dako Autostainer Link 48 is about one minute, 30% less than that of the Leica and Ventana systems. This suggests that the Dako system has greater efficiency.

This study was not intended to take into account other variables in the staining process that could impact overall staining times. Rather, the study used the standard methods provided by Dako and other vendors. There are obviously ways in which all systems could produce stains in less time. In any event, the new version 3.1.1 software is a significant advancement for the Dako Autostainer Link 48 stainer.

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General Methodology

Routine FFPE sections, cut at 3 μm , were collected on Dako charged slides (Code K8020, Dako Canada, Burlington, Ontario); these were air dried and further dried at 60 °C for 30 minutes prior to immunohistochemistry. Prior to immunohistochemistry, sections were either dewaxed in xylene or dewaxed as part of the antigen retrieval process. Offline dewaxing was comprised of three xylene changes (five minutes each), 3 rinses in absolute alcohol (approximately one minute each) and hydration through 70% alcohol to water. Other sections were loaded into the PT Link instruments and subjected to an antigen retrieval/dewaxing protocol which was comprised of increasing the temperature to 97 °C over a period of approximately 10 minutes; incubation at 97 °C for 20 minutes; cooling for an additional 10 minutes; and, finally, transfer to the Autostainer Link 48 instrument. Both high and low pH EnVision™ FLEX Target Retrieval Solutions (Dako, Burlington) were employed. For some antigens, sections were not pretreated or were subjected to trypsinization using 0.5% trypsin (Sigma, Toronto) in Tris-Buffered Saline (TBS) at pH 7.6, containing 0.5% calcium chloride, for 30 minutes at 37 °C before loading on the Autostainer Link 48.

Actual immunostaining was done with Dako primary antibodies. These were primarily Dako FLEX Ready-to-Use format, but some concentrates were also diluted in the laboratory with Dako Antibody Diluent (Dako, Burlington). The Dako EnVision™ FLEX Detection system was then employed, without linker antibodies, according to standard protocol times as follows: peroxidase blocking reagent: 10 minutes; primary antibodies: 20 – 30 minutes; detection system: 20 minutes; chromogen (diaminobenzidine; DAB): 10 minutes. At the end of the staining run, slides were flooded with distilled water and then manually counterstained with hematoxylin, dehydrated and coverslipped with a Sakura SCA tape coverslipping instrument (Sakura, Torrance, CA).

Detailed records of manual staining steps were kept for each staining run, and reports from the PT Link incubations and Autostainer Link 48 runs were used for analysis. Trained observers (ECC and RHS) performed microscopic evaluation of staining results. Bar charts and statistical analysis were created with GraphPad Prism software (San Diego, CA). All methods followed standard operating procedures established in the Gamma-Dynacare Molecular and Cellular Pathology Laboratory in Bowmanville, Ontario, Canada.

Comparison of staining times using software versions 3.0 and 3.1.1

In this phase of the study, direct run times of the two software versions on Autostainer Link 48 instruments were compared. Only the staining processes completed on the Autostainer Link 48 were compared. Run times were recorded manually and by the staining system software. Predicted run times, displayed by the software prior to initiating each run, were also recorded.

Routine surgical cases and controls were employed. Each block had two slides taken for each test, one for each instrument (two different software versions). Recognizing that total run time would depend upon the numbers of slides stained, a total of 23 experiments were conducted with varying numbers of slides. For analysis purposes, these experiments were divided into runs of 1 to 9, 10 to 19, 20 to 29, 30 to 39 and 40 to 48 slides. The *n* for each group was 4 or 5. Each pair of slides was loaded into identical locations on each instrument, to ensure that differences in robot travel time were negated.

In 23 experiments, more than 500 surgical tests/controls were run in duplicate. Stains were compared using either standard glass slides or, in some cases, digital slides produced by the Leica SCN 400 Slide Scanner (Quorum, Guelph, Ontario, Canada). Pairs of slides were categorized as having equivalent staining or as one slide being stronger than the other. In no case was the staining difference greater than 0.5 (on a scale of 0 to 3+).

Consistency of staining

Due to the nature of histological sample preparation, variations in staining intensity are to be expected when comparing multiple sections from the same block stained according to the same protocol. For example, while every effort is made to produce sections of the same thickness, a section that is slightly thicker or thinner than the average will occasionally be stained. In previous (unpublished) studies from the authors' laboratory in which batches of 30 or more slides from the same block were stained in parallel, there were clearly some differences in staining intensity. Nonetheless, in an ideal situation, sections stained by an automated system should display the same staining intensity as well as identically distributed immunoreactivity. Accordingly, it was important to assess any staining variation with sections immunolabeled by the new version 3.1.1 software.

Four sets of 48 slides were prepared. For two experiments, large slices of tonsil or placenta were employed and each slide held a single section. In the other two experiments, large blocks of liver were used, and two sections were collected to cover as much of the slide as possible. Vimentin was chosen as the test antigen and was labeled using the Dako Ready-to-Use anti-vimentin (Code IR630) and the EnVision™ FLEX detection system.

Overall staining was assessed at both macroscopic and microscopic levels to determine whether there were any variations within the slide, from station to station, or from run to run. Any smaller patch of weak or no staining, in any of the slides, was furthermore noted.

Additional studies

■ Reduced scanning time

The authors noted during previous experiments that scanning of slides and reagents appeared to be faster when using software version 3.1.1 compared to software version 3.0. Accordingly, 10 parallel runs of the same tests were performed on two instruments running software versions 3.0 and 3.1.1. The number of slides per run ranged from two to 46. Total scanning time was recorded manually for both instruments.

■ Lower buffer usage

Previous experiments indicated that buffer volume was lower on instruments running software version 3.1 than those running version 3.0. Therefore, formal comparisons of buffer usage were performed at the same time as scanning time measurement.

To determine buffer usage, bulk containers were marked with a volume scale by adding successive one-liter aliquots of buffer and indicating the fluid level with a magic marker. The estimated buffer consumption was then determined by comparing the starting volume to the ending volume. It is recognized that these are not accurate measurements, and they were rounded to the nearest 100 mL.

■ Less waste

Lower buffer usage also means less waste. The Autostainer Link 48 instrument has two waste outlets: one for hazardous waste and one for non-hazardous waste. Accordingly, two experiments were run using the instruments with the different software versions and full runs of 48 slides. The same tests were performed on each station of both instruments. Waste bottles were marked as described above, and waste levels were assessed visually. Again, these were not considered to be accurate and were rounded to 100 mL.