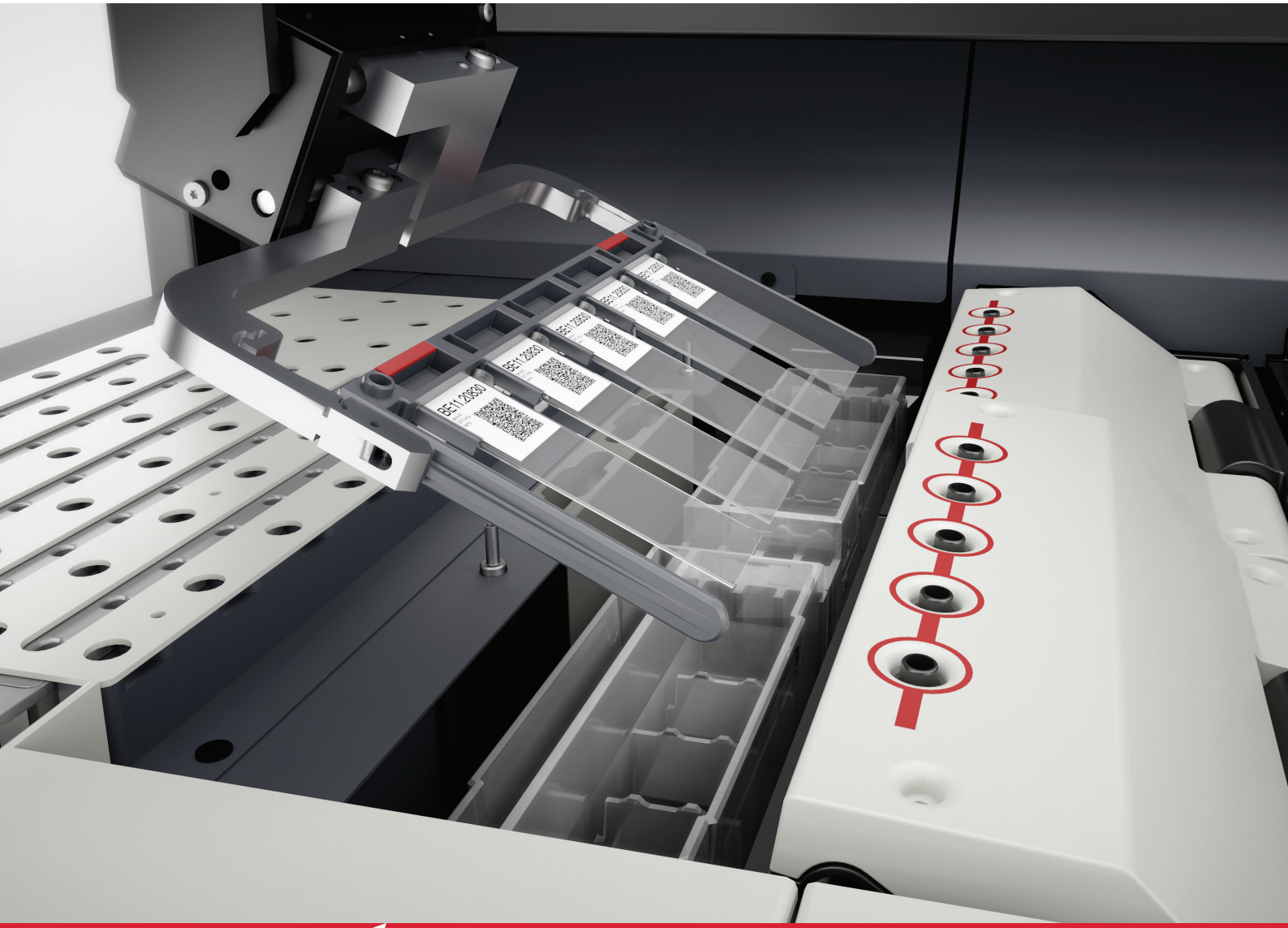
WHITE PAPER

Dako Omnis | Dynamic Gap Staining

Dako
A Better Path™

Dynamic Gap Staining
A New IHC Staining Technology



Agilent Technologies

Summary

Four different tests using immunohistochemical (IHC) staining on a total of 2,136 tissue sections were used to examine the repeatability and reproducibility of the Dynamic Gap staining technology employed in the newly introduced Dako Omnis automated staining solution. Analysis of staining intensity variation for slides stained in the same run, on different instruments and on different days showed that both intra-run and inter-run variation was low with an coefficient of variation of only 3% and 7% for both high expression and low expression structures, respectively. This study confirms that the largest contribution to overall staining variation is intra-run, whereas inter-day and inter-instrument variations contribute less to the overall variation, with instrument differences contributing the least. During the design of the Dako Omnis solution, there was a strong focus on preventing common staining artifacts such as patchy staining, edge effects and air bubbles. The Dynamic Gap staining technology has overcome these staining artifacts with none of 56 closely examined tissue sections showing any signs of the aforementioned artifacts.

Staining Principles

The first automated devices for immunohistochemistry appeared in mid-late 1980s, utilizing a number of different technologies (1-3). A key driver for implementation of automation was to avoid the labor-intensive, and therefore expensive, manual staining. Automation of IHC quickly accelerated, and in the mid 1990s, several IHC instruments were commercially available. Automated IHC staining has progressed since its introduction, with three types of IHC staining principles common in instrument systems in the last 10-15 years (Figure 1).

Open Individual Slide Staining

Slides are positioned horizontally, with reagents dispensed to one or more zones on the slide. Of the different principles, Open Individual Slide Staining mimics manual staining the most. The principle has been and is currently used on a number of slide stainers, including Autostainer Link 48 from Dako and intelliPATH from Biocare Medical.

Liquid Overlay Technology:

An inert fluid is deposited over the entire slide, and reagents are either overlaid with or deposited into the overlay fluid. Airstreams provide some level of reagent mixing on the slides, and reactions are carried out at an elevated temperature, facilitated by the overlay fluid that limits evaporation. Following staining completion, the slides need to be cleaned of the overlay fluid. This principle is currently used by Ventana Medical stainers.

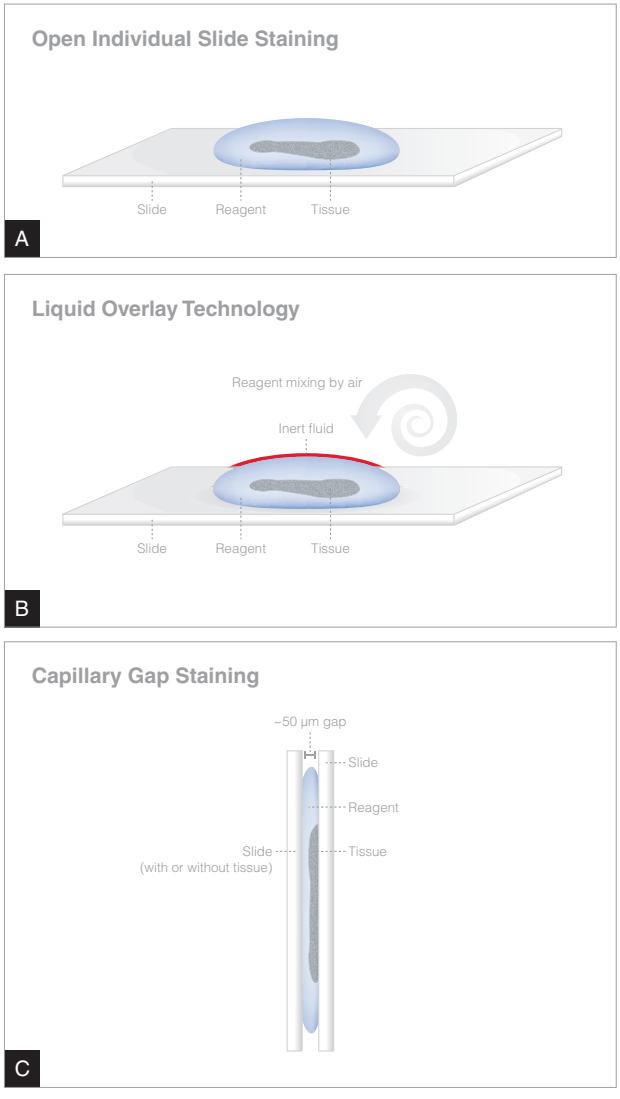


Figure 1: Traditional IHC staining principles. **A)** Open Individual Slide Staining, **B)** Liquid Overlay Technology, **C)** Capillary Gap Staining

Capillary Gap Staining

The capillary gap technology uses capillary forces to draw up and keep liquid between two planar units that may be either two microscope slides with tissue facing each other or a slide and a cover plate. This requires a narrow, definite spacing between the two units to facilitate capillary force across the entire slide. The capillary gap principle has been and is used on a range of stainers, including the TechMate instruments from Dako and the Bond series of instruments from Leica Biosystems.

The three staining principles each have their own advantages and drawbacks. For a comparison of several commercial stainers, please refer to review by Meyers (4).

Dynamic Gap Staining

To overcome drawbacks with current staining principles and to ensure consistent, high-quality IHC staining, a new staining technology, called Dynamic Gap, has been developed for and implemented in the Dako Omnis instrument. Dynamic Gap staining uses capillary forces during reagent application, while switching to continuous movement of reagents during reagent incubation and washes (Figure 2).

The capillary gap ensures that all reagents are distributed evenly over the entire slide surface, while the lid movement facilitates efficient reagent mixing and ensures homogeneous reaction conditions across the entire staining area during incubation. The cyclic motion during the washing steps ensures efficient and controlled wash conditions. The use of coverlids, combined with high humidity in the staining chambers, reduces evaporation so incubations can be performed at an elevated, constant temperature of 32 °C, which further ensures homogenous staining conditions and reproducibility between slides.

Performance of Dynamic Gap Staining

To investigate the performance of the Dynamic Gap staining technology, a series of tests were performed on Dako Omnis. In total, 798 slides and 2,136 tissues were stained to:

- Investigate repeatability and reproducibility between runs, instruments and days
- Test the uniformity of the staining quality over the full staining surface
- Test for common staining artifacts such as edge effects, patchy staining and air bubble formation.

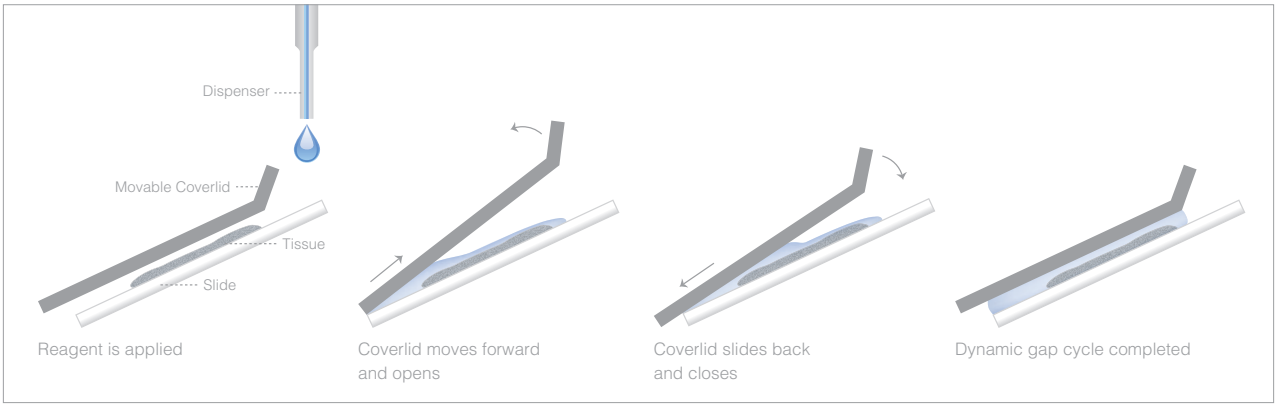


Figure 2: The glass slide with tissue is positioned at an angle of 25° to facilitate rapid reagent distribution across the slide. During reagent application, the coverlid creates a capillary gap to ensure homogeneous spreading of reagents throughout the entire staining area. When reagents are applied, the coverlid is placed with a slight overhang. During reagent incubation and washes, the lid moves in two directions. First, the lid moves forward. The upper end of the lid is then moved up, slowly at first to protect the tissue and bound reagents, and then at a higher speed to effectively create turbulence. When the lid is open, a reagent overlay protects the tissue from drying. Subsequently, the lid closes and moves back to the initial position. Movement is continuous during all incubations in cycles of 16 seconds each.

For the initial test, a panel of 14 antibodies was used to label antigens with different cellular localization, different antigen retrieval requirements and different needs for signal amplification in the visualization protocol. Tissue samples used were tissue microarrays (TMAs) or clinical cancer tissue according to the antibody's target. Similar testing was done for a panel of 30 Ready-To-Use (RTU) antibodies on high expression (HE) and low expression (LE) structures in normal tissue (Figure 3), as well as relevant clinical cancer tissue. Finally, two smaller tests were set up to test specifically for uniformity of the staining and common staining artifacts (Table 1).

Analysis of Stained Slides

All slides were scored on a staining intensity scale from 0 to 4 with 0.25 grade intervals (Table 2 and Figure 4) with score = 4 representing the highest intensity. Staining intensity was scored for both HE and LE structures as defined for each antibody in the Dako Atlas of Stain (5). For heterogeneous staining, an average score was given. In Test 1, 3 and 4 (Table 1), slides were scored blindly, while in Test 2 all slides stained with a specific antibody were evaluated by the same technologist in a comparison assessment.

Repeatability and reproducibility were evaluated based on differences in scoring intensity between serial sections on different slides. Uniformity of staining was evaluated based

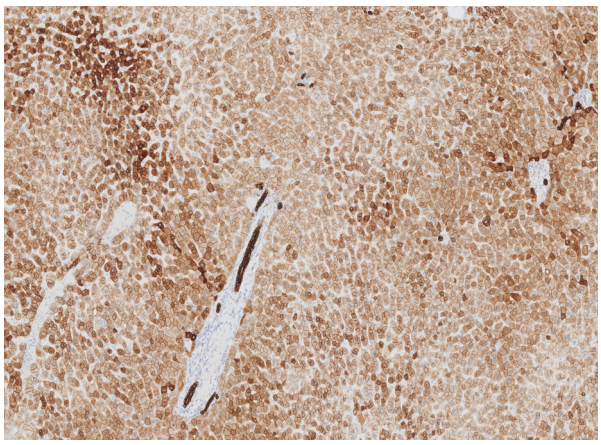


Figure 3. High (HE) and low (LE) expression structures in liver tissue. High expression and low expression structures are defined specifically for each marker. It is the relative staining intensity of the specific marker in two different cell types, tissue types, localizations, etc. that defines high and low expression structures. One marker can have a staining intensity score of 2.0 (Medium) in liver cells, but liver cells are still the LE structure since the same marker has a 3.75 (Very high) score in bile duct epithelial cells in liver. That means the bile duct epithelial cells are the HE structure.

Table 1. Experiments were conducted using the setup in this table. All tests were performed with FLEX IHC Microscope Slides or Superfrost Plus glass slides using FLEX Target Retrieval Solution, High pH or Low pH and EnVision™ FLEX or EnVision FLEX+ as visualization system.

Test	Antibody	Tissue thickness	Tissue type	No of slides/tissues	No of Instruments	Non-consecutive days
1	Antibody panel*	4 µm	TMA**	210/420	3	3
2	30 Omnis RTUs	4 µm	Multiblock***	540/1620	3	3
3	CK AE1/AE3	4 µm	Liver	20/40	1	1
4	Antibody panel*	4 µm	TMA	28/56	1	1

*Dako FLEX RTU antibodies AMACR, BCL2, CD3, CD7, CD20, CD45, CD68, CDX2, CEA, CK7, CK20, CK AE1/AE3, Ki-67, S100.

**TMA with sections from the following 12 tissues: tonsil 1 and 2, normal liver, breast carcinoma, lung carcinoid tumor, colon adenocarcinoma, malignant melanoma, normal colon, normal prostate, normal cerebellum, normal kidney, pancreas.

*** For each antibody, three different structures (HE structure, LE structure, clinical tissue) were analyzed.

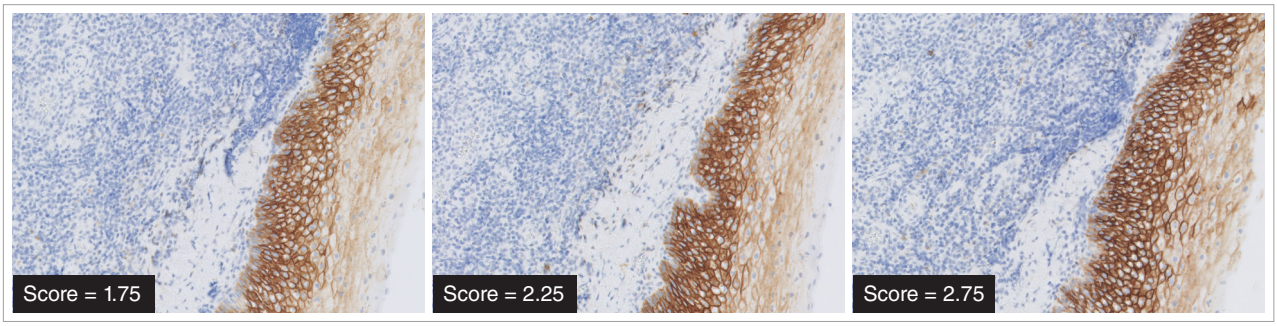


Figure 4: Scoring differences are exemplified showing three stains using Anti-CD138 on normal tonsil tissue with staining intensity scores as indicated.

on the differences in staining intensity in serial sections placed at the top and bottom of the same glass slide. Patchy staining was defined as areas in the same tissue with a difference of > 0.5 grade score, while air bubbles and edge effect were evaluated as either present or not present.

Repeatability and Reproducibility

Repeatability and reproducibility were tested using three Dako Omnis instruments tested over three non-consecutive days. A total of 210 slides were stained, each with tissue sections at the top and the bottom position; thus in total 420 tissue sections were stained and analyzed (Table 1, Test 1). The test was designed to address repeatability and reproducibility for: A) intra-run variation originating from slides and tissue quality, rack positions, staining modules, pre-treatment modules, and B) variation due to different days and different instruments.

Table 2. Staining intensity scoring. The scoring intensity is divided into five categories. Assessors were trained to be able to distinguish the minute differences in staining intensity in 0.25 intervals.

Score	Assessment
0	Negative
0.25 – 1.0	Low
1.25 – 2.0	Medium
2.25 – 3.0	High
3.25 – 4.0	Very high

Six of the antibodies (CEA, CK7, CK20, CD68, CDX2, CK AE1/AE3) showed no variation at all for HE structures (Figure 5). For the 14 antibodies, the difference in staining intensity from slide to slide, including both intra- and inter-run variations, shows very little variation with an average coefficient of variation of only 3% and 7% for HE and LE structures, respectively. The highest standard deviation (SD) was 0.24 for HE structures (AMACR) and 0.22 for LE structures (CDX2). Further analysis of the data revealed that all variation comes from intra-run parameters, i.e. the small variations that were observed originate from rack positions, staining modules, pre-treatment modules and slides, among other things. There was no observable variation between different instruments or on different days.

Test 2 was conducted with 30 FLEX Ready-to-Use antibodies developed and validated for use on Dako Omnis (Table 1, Test 2). For each antibody, three different structures (HE structure, LE structures and relevant clinical tissue) were stained in duplicate on three different, non-consecutive days on three different instruments, meaning that a total of 54 individual tissue structures were stained and analyzed for each antibody.

The results showed a very low degree of variation (Figure 6) with no SD above 0.17, which is less than the scoring resolution. This study confirmed that the highest contribution to variation was intra-run differences. Instrument differences contributed the least to the variation.

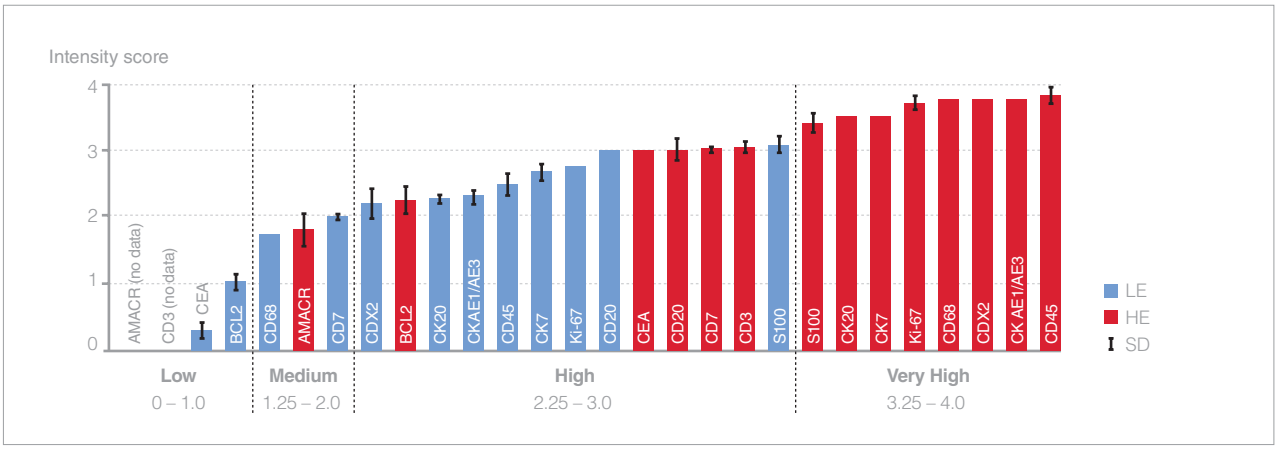


Figure 5: Average staining intensity score for the 14 antibodies in Test 1. Standard deviation (SD) is indicated by black bars. Please notice that for some antibodies, e.g. CD20 and S100, the low expression structures give staining intensity scores that are termed "High", but these structures are still termed LE, since other tissue structures give even higher staining intensity.

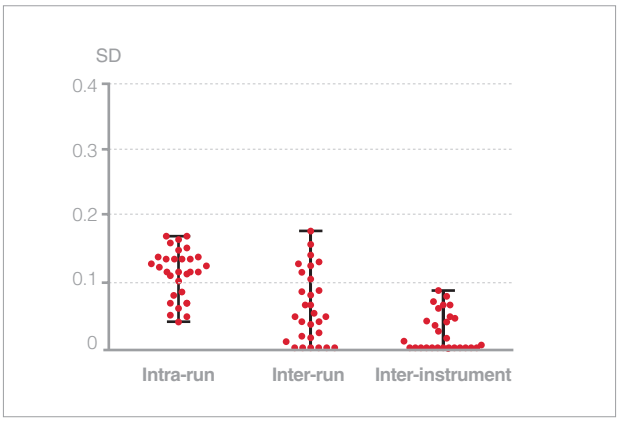


Figure 6: Standard deviation (SD) and range of variation in staining intensity from tissue scored on the 0-4 staining intensity scale. Intra-run variation has the highest impact on variation. The highest observed SD is 0.17 and thus below the resolution on the scale showing very little difference in staining intensity. Each dot represents an SD data point. The black bar represents the SD range.

The combined set of data from Test 1 and Test 2 demonstrates the capability of the Dynamic Gap staining technology to produce consistent staining on the Dako Omnis instrument, with little variation in staining intensity between different instruments and on different days (Figure 7). This data strongly supports the reproducibility of staining on the instrument, with the largest contribution originating from intra-run variation. However, even the intra-run variation is lower than the smallest interval (resolution) on the intensity scale.

Uniform Staining Anywhere on the Glass Slide

Most laboratories occasionally experience uneven staining intensity depending on the location of the tissue section on the glass slide with their existing stainer(s). To analyze whether Dynamic Gap staining can produce uniform staining across the total staining area, tissue sections were placed in both the top and bottom positions (Figure 8) on 210 glass slides (Test 1).

For HE structures, 89% of the slides did not show any difference in top-bottom staining intensity. On the 0-4 staining intensity scale, a difference of 0.25 between top and bottom was found for HE structures in 10% of the slides while a maximum difference of 0.5 was found for 1% of the slides. Assessment of LE structures showed comparable results; 89% of the evaluated slides containing LE structures showed no top-bottom difference and less than 1% of slides had a maximum difference of 0.5 staining intensity from top to bottom (Figure 9).

To test staining efficiency in the outermost areas of the glass slide, an experiment was designed (Test 3) where tissue sections were positioned at the outer corners of the staining area as illustrated in Figure 10. All slides were stained with Anti-Cytokeratin, Clone AE1/AE3, on liver tissue. Again, very low difference in staining inten-

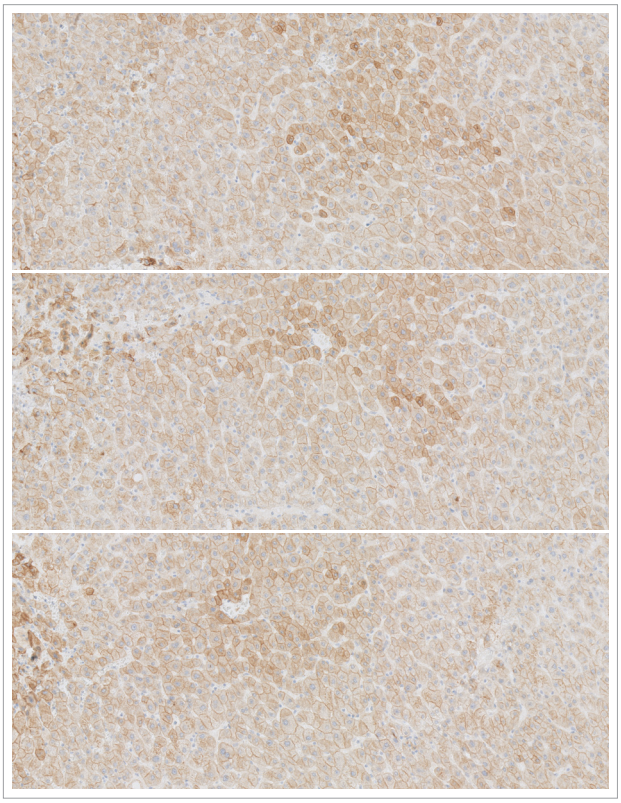


Figure 7: Liver tissue stained with Anti-CK, Clone AE1/AE3 showing consistent staining intensity. Sections from same normal liver tissue block was stained on the same instrument on three different days.

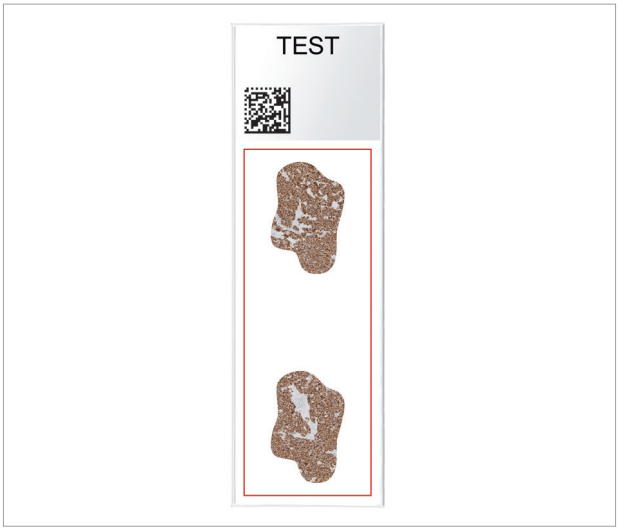


Figure 8: Illustration of top and bottom positioning of tissue sections on a glass slide.

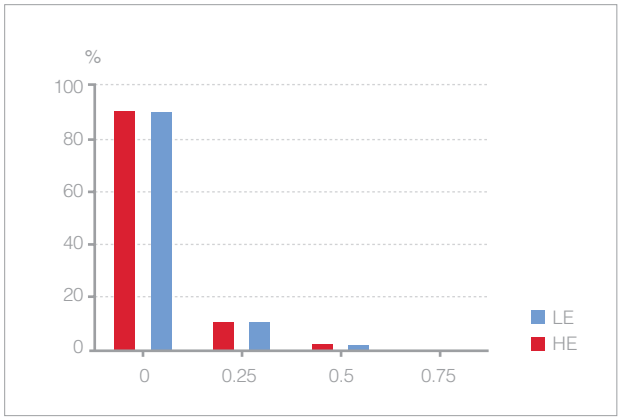


Figure 9: Percentage of slides showing 0, 0.25 or 0.5 difference in staining intensity between top and bottom positioning of tissue sections on the glass slide stained on Dako Omnis.

sity was observed for both HE and LE structures with 93% of the slides showing a difference of 0.25 or less.

The cyclical movement of the coverlid is an essential part of the Dynamic Gap staining technology. This motion gives an effective mixing of reagents as well as an effective wash between incubation. At the same time, the Dynamic Gap ensures that the reagent is effectively moved across the entire staining area. Combined with the Dako reagents and optimized staining protocols, Dynamic Gap staining practically removes top-bottom and corner area differences.

The consistent, homogenous staining across the entire staining area gives full flexibility of tissue section positioning within the staining area, and enables even very large tissue sections to be stained uniformly. This is particularly relevant when staining control tissue, which is often placed at the top end of the slide.

Common Staining Artifacts Effectively Removed

Patchy Staining

Patchy staining may be caused by a number of different factors, including insufficient removal of paraffin, drying of tissue following target retrieval or during the staining process and by local reagent depletion. Patchy staining

(Test 4) was assessed in 56 tissue sections stained with the same antibodies as used in Test 1, and patchy staining was not observed in any of the tissue sections. This indicates that Dynamic Gap staining does not create artifacts caused by, for example, reagent depletion as sometimes seen with other staining principles. In particular, immunohistochemical staining of CD20 and CD45 are susceptible to patchy staining due to reagent depletion, but the Dynamic Gap staining technology gives crisp, homogenous staining throughout the slides (Figure 11).

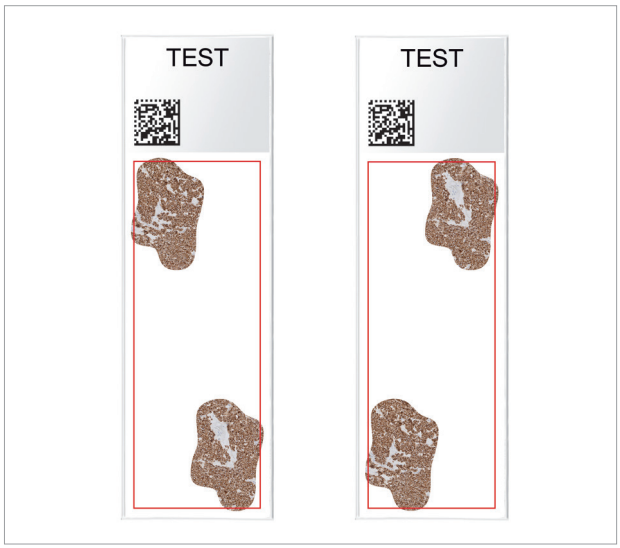


Figure 10: Positioning of tissue on slides in Test 3 to cover all four corners of the staining area. Tissue samples were positioned to facilitate assessment of staining quality at the border of the staining area, as indicated by the red box.

Edge Effect

Edge effect is typically seen as a distinct change in staining intensity from positive to very weak (or negative) within a very short distance, typically at the edge of the tissue. The primary reasons for this effect include drying of tissue and uneven distribution of one or more of the staining reagents. No edge effect was observed for any of the 56 tissue sections analyzed in Test 4.

Air Bubble Formation

Impact on staining quality from presence of air bubbles during reagent incubation was also registered. No slides were found with signs of air bubble formation. Furthermore, there have been no observations on impact on

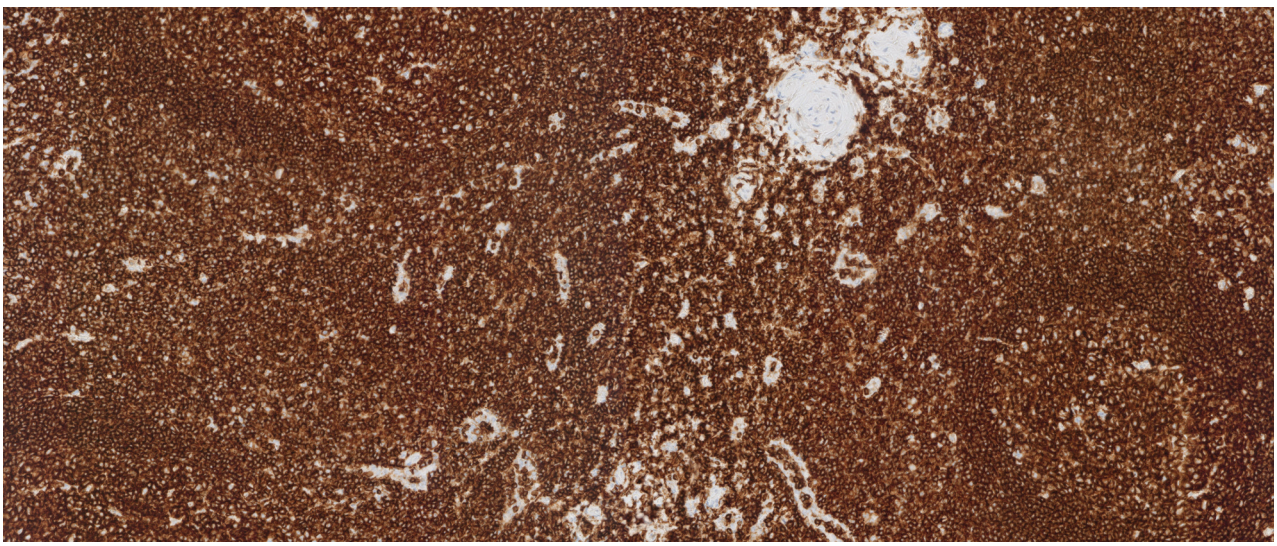


Figure 11: Tonsil stained with Anti-CD45 on Dako Omnis. Intense staining without any sign of depletion was seen for all antibodies, here exemplified with Anti-CD45 which may show patchy staining due to reagent depletion in other automated staining principles.

staining quality originating from air bubbles in any testing done on Dako Omnis.

In order to address the risk of air bubble formation, the Dynamic Gap staining technology was designed with slides positioned at a 25° angle to facilitate escape of air bubbles that may be present in the reagents during application. To further prevent interference from air bubbles, bulk reagents pass through an 'air bubble trap' designed to remove air. Video recordings (not shown) of the staining procedure from reagent application to washing showed that when bubbles were deliberately forced into the system, they were effectively removed during the first movement cycle of the coverlid. Bubble formation and related effects on staining quality can thus be avoided with Dynamic Gap staining, due to the continuous mixing of reagents as well as the slide angle of 25°, in which all incubations and washes are conducted.

Conclusions

There are many factors that may introduce variations in immunohistochemistry: the tissue itself being a biological material with natural variation, a range of pre-analytic factors including ischemic time and fixation conditions, and a range of analytical factors including the antibody specificity and sensitivity, antigen retrieval, staining protocol – and not least the instrument performing the staining processes.

To obtain consistent, high-quality staining results it is important to optimize and standardize whenever and wherever possible. The Dynamic Gap technology has been developed to support uniform staining across the entire slide, and it is fully explored through the integration with the Dako Omnis staining solution working optimally with the RTU FLEX reagents and validated staining protocols.

A prerequisite for high-quality staining is ensuring intra-run repeatability and inter-day/inter-instrument reproducibility. Staining intensity variation on Dako Omnis is very low with intra-run contributing the most to the variation. Thus, the staining intensity is similar for serial tissue sections stained on different instruments and on different days. The variation is lower than the resolution on the intensity scale and so low that we would claim that it is challenging to see the difference for the untrained human eye. The Dynamic Gap staining technology thus supports consistent and reproducible staining both between slides in the same run, between instruments and on different days.

During design of the Dako Omnis solution there was a strong focus on preventing staining artifacts that have been observed when using some of the current staining principles. These staining artifacts include top-to-bottom differences, patchy staining, edge effects, and air bubbles. The Dynamic Gap technology has overcome the factors that generate these common staining artifacts. The capillary forces that form during reagent application ensure homogeneous spread over the entire staining area, and the 25° position of the glass slides facilitate the escape of air bubbles that may be present in the reagents during application.

The goal when designing the Dynamic Gap technology was to standardize the staining environment to support consistent, uniform, high-quality staining results. From the test results, it is evident that it provides consistent staining results with very low variation between slides, instruments and days.

We believe the Dako Omnis solution has reached the goal.

References

1. Stark E, Faltinat D, Von der Fecht R. An automated device for immunocytochemistry. J Immunol Methods 1988;107:89-92.
2. Tubbs RR, Bauer TW. Automation of immunohistology. Arch Pathol Lab Med 1989;113:653-7.
3. MaWhinney WHB, Warford A, Rae MJL, Lauder I. Automated immunohistochemistry. J Clin Pathol 1990;43:591-6.
4. Myers J, A review of automated slide stainers for IHC and ISH. Med Lab Observer 2008;Jan:41-4.
5. Atlas of Stains, 4th edition. FLEX Ready-to-Use. Dako handbook. 2013.

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