Agilent
Dako

CE

General Instructions
For Primary Staining

Purpose
These general instructions apply to Hematoxylin and Eosin (H&E) Primary Staining reagents from Agilent. Always consult the specific Instructions for Use for your product for specific instructions on the use of the product.

The information in this General Instructions for Primary Staining are guidelines only. A validated protocol is provided in the specific Instructions for Use for each reagent together with the Dako CoverStainer User Guide. Optimal procedures must be determined and verified by the user.

NOTE: Contact Agilent Pathology Support via www.agilent.com to report any unusual staining. Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the country in which the user and/or the patient is established.

Principle of Procedure
The principle of the H&E staining procedure is the sequential application of two different stains. First is deparaffination in xylene or xylene substitute, Histo-Clear II is recommended, followed by hydration in declining concentration of ethanol ending up in water before application of the first stain hematoxylin. The staining component is hematein, which is the oxidation product of hematoxylin, combined with a mordant (metallic salt). Hematein forms a cationic (positively charged) complex with the mordant, which enables chelation with negatively charged binding sites of cell components, which are most abundant on chromatin located in the nucleus. If a progressive staining procedure is used, the hematoxylin is stained to the desired intensity. In a regressive staining procedure, the tissue is first overstained with hematoxylin, followed by a rinse in Differentiation Solution to remove excess staining (differentiation). Bluing comes next, which converts the initial soluble red color of the hematoxylin to an insoluble blue color. Counterstaining with the second stain - eosin - follows. Eosins are anionic (negatively charged) enabling staining in various shades of pink of most proteins in almost all the structures present in any tissue. Then follows rinse in ethanol for differentiation of the eosin and finally permanent mounting. As a result, nuclei are stained blue to purple and cytoplasm, collagen and red blood cells are stained in varying shades and intensity of pink, orange, and red. [1–3]

The user should read these General Instructions and product-specific Instructions for Use and the User Guide for the Dako CoverStainer instrument carefully to become familiar with all the components and the instrumentation prior to use.

Materials Required, but Not Supplied
See individual primary staining reagent's Instructions for Use for specific recommendation of procedure. Not all of the below listed materials may be required.

Equipment and accessories
Dako Coverstainer (Code CS100)
Dako Coverstainer Slide Rack (Code CS119)
Dako Cover Glass (Code CS704)
Microscope slides having nonbeveled edges (25–26 mm x 75–76 mm)
Light (bright field) microscope (4–40X objective magnification)
Other ancillary components, including instrument-specific ancillaries and accessories, may be necessary.

Chemicals, buffers, and solutions
Dako Hematoxylin (Code CS700)
Dako Eosin (Code CS701)
Dako Bluing Buffer (Code CS702)
Dako Mounting Medium (Code CS703)
Dako Toluene Free Mounting Medium (Code CS705)
Dako Differentiation Solution (Code CS707)
Dako Gill's 3 Hematoxylin (Code CS708)
Dako Harris Hematoxylin (Code CS709)
Dako Eosin Y Phloxine B (Code CS710)
Dako Modified Eosin Y (Code CS711)
Tap water
Demineralized or de-ionized water (reagent-grade quality water)*
99.9 % Ethanol
96% Ethanol
70% Ethanol
Xylene or xylene substitutes, Histo-Clear II is recommended

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Controls
Tissue controls

*Note: Not all sources of water may be of sufficient quality for H&E staining. It is recommended to use reagent-grade water corresponding to Clinical Laboratory Reagent Water (CLRW) standard as specified by CLSI, or water similar in quality to be used.

Storage
Store at room temperature (15–30 °C). Do not use after expiration date. Dako Hematoxylin (Code CS700), Dako Eosin (Code CS701), Dako Bluing Buffer (Code CS702), Dako Differentiation Solution (Code CS707), Dako Gill's 3 Hematoxylin (Code CS708), Dako Harris Hematoxylin (Code CS709), Dako Eosin Y Phloxine B (Code CS710) and Dako Modified Eosin Y (Code CS711) have an onboard stability of 5 days, if drained back into the bottle on a nightly basis. These reagents should be used within 5 days or replaced after 3000 slides (depending on what comes first). The hematoxylins shall be stored in the dark. The onboard stability for Dako Mounting Medium (Code CS703) and Dako Toluene Free Mounting Medium (Code CS705) is 5 days. If products are stored under any conditions other than those specified in the Instructions for Use, they must be validated by the user.

Reagents for H&E from Agilent should be stored according to the conditions detailed in their Instructions for Use. Reagents should be discarded according to local Health and Safety regulations. Reagents are suitable for use until the expiration date printed on the product label, when stored at conditions specified on the product label and in the Instructions for Use. See Instructions for Use of individual primary staining reagents for onboard stability on Dako CoverStainer.

There are no obvious visual signs to indicate incorrect product storage or handling of this product during the product's shelf life. If a problem is suspected with the product that cannot be explained by incorrect product storage or handling, or other variations in laboratory procedures, contact Agilent Pathology Support. Refer to the Troubleshooting sections for more information.

Specimen Preparation
These are guidelines only. Optimal procedures must be determined and verified by the user.

Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection, and disposed of with proper precautions.[4] Specimens must be handled to preserve the tissue for H&E staining. Standard methods of tissue processing should be used for all specimens.[1,5] Prior to H&E staining, tissues must be fixed and processed. Fixation prevents autolysis and necrosis of excised tissues, preserves antigenicity, enhances the refractive index of tissue constituents and increases the resistance of cellular elements to tissue processing. Tissue processing includes dehydration, clearing of dehydrating agents, infiltration of embedding media, embedding and sectioning of tissues. The most common processes for tissue and cell preparations are discussed below. Agilent Dako reagents for H&E have been validated with paraffin-embedded tissues fixed in 10% formalin (FFPE), frozen sections, and cell preparations.

Paraffin-Embedded Tissue
General comments
The most common tissue fixative is 10% (v/v) neutral phosphate-buffered formalin, commonly referred to in the EU as 4% w/v buffered formalin. It is important to maintain optimal, standardized fixation conditions whenever possible in order to obtain reproducible staining, which may depend on the type and concentration of fixative, on fixation time, and on the size of the tissue specimen to be fixed.

Tissue fixation in formaldehyde-based solution (neutral buffered formalin)
Most formaldehyde-based fixatives contain 10% formalin, a neutral salt to maintain tonicity, and a buffered system to maintain pH. These fixatives are well tolerated by tissues and exhibit good histological penetration. However, shrinkage or distortions may occur in poorly fixed and embedded tissue specimens. Fix small blocks of tissue (approximately 10 x 10 x 3 mm) in a 1:20 volume ratio of fluid to tissue,[5] with a fixation time for 12–48 hours will generally show good cytoplasmic preservation and nuclear detail.[3,6] Under-fixation may compromise staining result. Prolonged exposure to fixatives may result in the cross-linking methylene bridges formed between two close formaldehyde binding sites, causing lowered permeability to macromolecules but the structures of protein molecules are not greatly altered. Prior to staining, dehydrate, clear, and embed tissue sections as described below in Processing and paraffin-embedding.

See references 1, 2, 5 and 6 the primary staining reagent's Instructions for Use(s) and the protocol(s) supplied with the fixing reagent(s) for additional information regarding tissue fixation.

Processing and paraffin-embedding
After fixation, processing may be completed using an automatic tissue processor. Tissues are dehydrated using graded alcohols, cleared with xylene or xylene substitute, and infiltrated with paraffin wax. The tissue is subsequently embedded with paraffin wax in molds or cassettes that facilitate tissue sectioning. Do not expose tissues to temperatures in excess of 60 ℃ during processing. Tissue blocks may be stored or sectioned on completion of embedding.

Adherence of paraffin-embedded tissue sections to microscope slides
Collect sectioned tissues (4–5 µm) from paraffin-embedded blocks on clean glass slides.

Frozen
Frozen sections should be cut into sections of 5 µm and air-dried for minimum one hour at RT. Dried sections can be stored at –20 °C until used. Before H&E staining, frozen tissue sections should be fixed in precooled acetone for 60 seconds (acetone kept in freezer at –18 °C overnight prior to use). Slides should be stained immediately after fixation to avoid drying out. Can be fixed in room temperature (20–25 °C) acetone for 10 minutes or in buffered formyl-acetone for 30 seconds.

Cell preparation
Preservation of cells is usually accomplished by immersion in a fixative fluid or merely drying the preparation acts as a form of preservation. The fixatives employed prevent autolysis by inactivating lysosomal enzymes, and they stabilize the fine structure, both inside and between cells, by making macromolecules resistant to dissolution by water and other liquids. Fixatives also inhibit the growth of bacteria and molds that give rise to putrefactive changes.[6]

Deparaffinization and rehydration
Prior to staining, tissue slides must be deparaffinized using xylene or xylene substitutes to remove embedding media and rehydrated in a regressive series of ethanol. This process is performed automatically onboard Dako CoverStainer.

Xylene or xylene substitutes should be replaced according to the work routines of the individual laboratory. Xylene substitutes, such as the recommended Histo-Clear II, may be used in place of xylene.

The following reagents should be replaced daily on Dako CoverStainer:
- Bottle G: Replace with fresh demineralized/deionized water
- Bottle I: Replace with fresh demineralized/deionized water (only for Dako Progressive Staining protocol)
- Bottle N: Replace with fresh 96% ethanol

Rotate 99.9% ethanol for dehydration daily in the following way:
- Bottle O: Replace with bottle P
- Bottle P: Replace with bottle Q
- Bottle Q: Replace with fresh 99.9% ethanol

It is recommended to rotate Exit solvent daily in the following way:
- Bottle R: Replace with bottle S
- Bottle S: Replace with bottle T
- Bottle T: Replace with fresh Exit solvent

Note: 99.9% ethanol (bottle O-Q) and Exit solvent (bottle R-T) are rotated in the described order to avoid that evaporation and contamination is not centered to one single bottle.

Xylene or xylene substitute (bottle A-B), 70% ethanol (bottle C-D), and 96% ethanol (bottle E-F) should be replaced according to the work routines of the individual laboratory, depending on number of slides processed, humidity, etc.

Read the User Guide for the Dako CoverStainer instrument carefully and become familiar with all the components and the instrumentation prior to use.

Consult *Education Guide: Special Stains and H&E, Second Edition* (6) or references 1, 2 and 5 for further details on specimen preparation for H&E staining.

Staining Procedure
Use the recommended reagents and the validated protocol provided in the Instructions for Use or Dako CoverStainer Basic User Guide for optimal staining performance on Dako CoverStainer.

NOTE: Any modification of the recommendations outlined in the Instructions for Use must be validated by the user.

Quality Control
Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results. Suitable tissue controls should be run daily. These quality controls are intended to ensure the validity of the staining procedure, including reagents, tissue processing and instrument. Controls should be biopsy/surgical specimens fixed, processed, and embedded in the same manner as the patient sample(s). If controls are not fixed in the same way as the test specimen, the tissue may only be used as a staining control for reagents and instrument performance.

Assay Verification
Prior to initial use of H&E primary staining in a diagnostic procedure, the user should verify the staining by testing it on a series of lab-supplied tissues.

These quality control procedures should be repeated for each new lot, or whenever there is a change in assay parameters. Known tissue processed in the same manner as the patient tissues are suitable for assay verification.

Stained Slide Storage
Some fading of stained slides may occur, depending on several factors including, but not limited to, slide storage conditions. To minimize fading, store stained slides in the dark at room temperature (20–25 °C).

Staining Evaluation
For evaluation of H&E, objectives of 4–40X magnification are appropriate. The entire tissue section should be considered, avoiding edge effects, necrotic areas, and areas with other obvious artifacts. Nuclei are stained blue to purple and cytoplasm, collagen, and red blood cells are stained in varying shades and intensity of pink, orange, and red.

General Limitations

1. H&E is a multistep diagnostic process that requires specialized training.
2. Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, incomplete paraffin removal or contamination with other tissues or fluids may produce artifacts. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue. Contact Agilent Pathology Support with any documented unexpected reaction.
3. The clinical interpretation of any staining must be complemented by proper controls and should be evaluated within the context of the patient's clinical history and other diagnostic tests. It is the responsibility of a qualified pathologist who is familiar with the reagents and methods used to interpret the stained preparation. Staining must be performed in a certified licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of tissue controls.
4. The reagents are ready-to-use, prediluted, and optimized for use with Dako CoverStainer staining system. When used in conjunction with other than the specified reagents and protocols these are no longer ready-to-use and the protocol must be reoptimized and validated according to the clinical laboratory's H&E validation protocol.
5. When properly stored, the reagent is stable to the date indicated on the label. Do not use reagent beyond the expiration date. Improper storage and use of reagents may lead to erroneous results.
6. The user should always ensure adherence to the maintenance schedule for the Dako CoverStainer instrument. Lack of adherence to the maintenance schedule may give erroneous results.

Troubleshooting

Refer to the Troubleshooting section in *Education Guide: Special Stains and H&E, Second Edition* (6) for a comprehensive guide to troubleshooting including remedial action.

Contact Agilent Pathology Support via www.agilent.com to report any unusual staining. Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the country in which the user and/or the patient is established.

Below is a list of common problems and solutions when using the H&E primary stain concept with recommended products and validated protocols.

Problem	Probable Cause	Suggested Action
1. Weak staining of slides	1a. Wrong storage conditions used for reagents.	1a. Check that reagents have been stored correctly according to recommended storage conditions.
	1b. Reagent is used past its expiration date.	1b. Ensure reagent is not used past its expiration date.
	1c. Reagent is used past its onboard stability of 5 days.	1c. Ensure reagent is not used past its onboard stability of 5 days.
	1d. Reagent is not drained back in bottle overnight.	1d. Ensure reagent is drained back in bottle overnight.
	1e. Reagent is used past the recommended 3000 slides.	1e. Ensure reagent is not used past the recommended 3000 slides.
	1f. Inappropriate fixation method used.	1f. Ensure that patient tissue is not fixed for too short or too long a time period, and that the correct fixative was used.
	1g. Incorrect placement of slides in Dako CoverStainer.	1g. Check placement of slides.
	1h. Specimen exposed to acidic solutions prior to staining (e.g. decalcification media, or unbuffered formalin or picric acid fixatives).[7]	1h. Do not expose specimen to acidic solutions prior to staining.[7]
	1i. Acid tap water.[6]	1i. Use distilled water.[6]
	1j. Chlorine in tap water.[6]	1j. Use distilled water.[6]
	1k. Dako Differentiation Solution used past recommendations in IFU.	1k. Follow recommendations in IFU.
2. Over staining of slides	2a. Inappropriate fixation method used.	2a. Ensure that only approved fixatives and fixation methods are used.
3. Uneven staining of slides	3a. Sections dried after staining procedure.	3a. Avoid sections drying out during the staining procedure.
	3b. Same xylene and ethanol used repeatedly.	3b. Replace with fresh xylene and ethanol.
	3c. Sections dried prior to coverslipping.	3c. Avoid stained slides drying out between staining and coverslipping.
	3d. Inappropriate fixation method used.	3d. Ensure that approved fixative was used. Alternative fixative may cause excessive background staining.
	3e. Paraffin residue.	3e. Replace xylene and ethanol.
	3f. Thermal artifacts in the tissue. Caused by electrocautery and laser excision or overheating of the wax[8].	3f. Avoid thermal artifacts.

References

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