

# HistoSonda

## TECHNICAL MANUAL





## HistoSonda Protocol

### Introduction

The HistoSondas are new single stranded DNA probes for chromogenic visualization of gene expression by in situ hybridization. The characteristics of these innovative new probes set them apart from those already on the market.

The HistoSondas consist of single stranded DNA fragments whose lengths are between 10 and 30 times greater than commercial oligo probes (the length of a HistoSonda is between 200 and 1500 nucleotides). The probe DNA is labeled with digoxigenin.

The probe DNA is targeted against RNA expressed by genes of interest and therefore the HistoSondas are probes for the detection of gene expression. The HistoSondas are specifically designed to be used on formalin-fixed and paraffin-embedded tissue sections.

The HistoSonda protocol is simple. It requires neither special apparatus nor working solutions different than those of standard immunohistochemistry.



To follow this manual or find out more specific details you can find and watch the Video Demonstration HistoSonda® Protocol at:  
<http://www.cenbimo.com/e-learning.html>

## IN COMPARISON TO MOST TRADITIONAL OLIGO PROBES THE HISTOSONDAS ARE:

### Faster

The HistoSondas® have an incubation temperature of 62°C. The elevated thermal energy generated allows the molecules to vibrate quicker therefore taking less time to find their target even though they are situated in a very viscose dextran containing media. The use of 62°C as the incubation temperature for these probes therefore permits rapidity. HistoSonda® is the only commercial probe whose incubation **period is just 60 minutes**.

### More specific

Due to their great lengths the HistoSondas® are more specific. It is statistically less probable to find shared sequence homology between long sequences. Therefore the chances of the probe annealing with anything other than the target sequence are greatly reduced, increasing specificity. The HistoSonda® incubation temperature of 62°C also permits that only chains with **almost total sequence homology are annealed** (an oligo will not anneal at this temperature).

### More potent

The potency of the HistoSondas® is directly influenced by the number of labeled nucleotides in the DNA sequence. As the HistoSondas® have long sequences, the probability of **large numbers of labeled nucleotides** being incorporated is greatly increased, increasing the potency of the signal.

## Simple to use

The HistoSonda® hybridization procedure has been simplified to the maximum. **It only uses one buffer** during the entire technique (PBS). **The use of formamide is not necessary** and post-incubation washes with formamide have therefore been suppressed. The hybridization procedure can be carried out without needing to have specific knowledge of Molecular Biology. Complicated apparatus is not necessary and revealing the probe can be performed automatically using any immunohistochemistry apparatus.

## Easy to transport and store

The Histosondas® are supplied Lyophilized in single use tubes. **Transport and storage can be carried out at room temperature** and they have a long storage life. Freezers, refrigerators or any special type of care are not required.

## Tissue requirements

Tissue sections must be formalin-fixed and paraffin embedded. Varying fixation/decalcification methods affect target retrieval by Proteinase K. The recommended protocols are indicated below:

### Fixation:

100ml Commercial Formaldehyde (37-40%)  
 900ml Saline solution (NaCl 9g/1000ml)

Fix for between 16-24hrs optimum.

### Decalcification:

100ml Commercial Formaldehyde (37 - 40%)  
 9g NaCl  
 50ml Glacial CH<sub>3</sub>COOH (Acetic Acid)  
 Fill until the 1 litre mark with distilled H<sub>2</sub>O.

Depending on the thickness of the cylinder, decalcify for between 24 and 48 hours.



Tissue sections should be cut between 4-6µm and adhered to silanized slides. The cut should be recent – ideally not more than 2 weeks old. Block age does not affect the reaction.

## Materials supplied by CENBIMO

- HistoSondas
- Proteinase K
- Primary Antibody Anti-Digoxin

## Materials required

- Incubator 62°C (it is important that this temperature be actual)
- Humid Chamber (62°C)
- Humid Chamber (room temperature)
- PBS pH 7.4 without Tween
- High quality distilled water
- Peroxidase-blocking solution (we recommend 0.3% H<sub>2</sub>O<sub>2</sub> in Methanol or 3% H<sub>2</sub>O<sub>2</sub> only)
- Secondary Antibody Polymer Anti-Mouse HRP / DAB revealing system
- Harris Hematoxylin
- Slide holders
- Wash baths
- Microwave or boiling distilled water
- Vortex and Centrifuge (where possible)
- General laboratory reagents for de-waxing paraffin embedded tissue sections.
- Mounting Medium and Cover Slips

## Additional materials required for HistoSonda DUAL

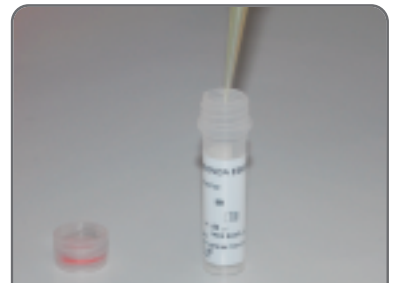
- TBS pH 7.4 without Tween (to avoid alkaline phosphatase interaction with PBS)
- Primary Antibody Anti-Biotin (Rabbit)
- Secondary Antibody Polymer Anti Rabbit AP/Fast Red revealing system

## Before you start...



**Pre-heat the humid chamber and incubator to 62°C.**

Reconstitute the probe by adding 65µl of high quality distilled water to the HistoSonda vial. Briefly vortex and centrifuge the tube where possible. If a cloudy precipitate remains in the solution place in an incubator at 62°C for 15mins to dissolve the precipitate.



Reconstitute the probe by adding 65µl of high quality distilled water.

## Dewaxing

Removal of paraffin and tissue preparation for hybridization, including a block for endogenous peroxidase.

### General considerations

It is possible to follow your normal dewaxing protocol without adverse effects. We include instructions here for guidance purposes, but it is important to block for endogenous peroxidase. We recommend a blocking solution of 0.3% hydrogen peroxide in methanol (e.g. 25ml 3% H<sub>2</sub>O<sub>2</sub> in 225ml Methanol) or 3% hydrogen peroxide only.

1. Place slides horizontally on a hot plate at 62°C or using a slide holder introduce into an incubator at 62°C for 10 minutes.
2. Immerse slides in the follow solutions:
  - Xylene 10mins
  - Xylene 5mins
  - Absolute alcohol (ethanol or isopropanol) 1min
  - Absolute alcohol (ethanol or isopropanol) 1min
  - Alcohol 96% 1min
  - Alcohol 96% 1min
  - Alcohol 96% 1min
3. Introduce slides into a blocking solution for endogenous peroxidase for 5 minutes.
4. Wash well with distilled water, agitating the slides for 30 to 60 seconds. Leave to stand in distilled water for 1 minute.



### Inhibition of unspecific DNA binding (optional)

Generally this step is not necessary unless the tissue to be hybridized contains a great quantity of polymorphonuclear eosinophil leukocytes (generally bone marrow and gastric tissues).

Due to its negative charge, eosinophils can unspecifically bind DNA resulting in an undesired labeling of these cells during the hybridization process. If the tissue to be hybridized is known to contain large numbers of eosinophils, unspecific labeling can be avoided by bringing the tissue sections to the boil in distilled water after peroxidase blocking and before deproteinization.

1. Place slides in 200ml distilled water in a microwave until the liquid boils uniformly (normally around 2mins).
2. Immediately transfer slides to distilled water at room temperature.
3. Alternatively place slides in boiling distilled water for 30 seconds and immediately transfer to distilled water at room temp.



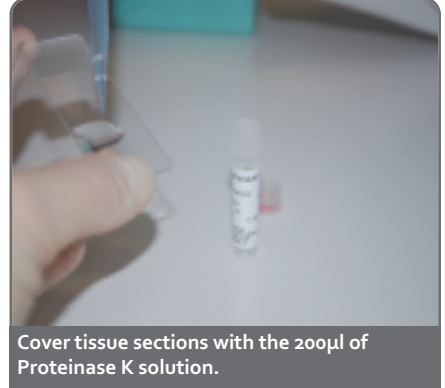
Excess heat treatment will result in background staining and damage to the tissue. Remove slides immediately when uniform boiling is observed. For guidance this is normally between 2-3 minutes when using an 800W microwave.

## Deproteinization

Target retrieval. Removal of RNA associated proteins such as ribosomes which impede the probe binding with its target.



Mix each vial of Proteinase K with 200µl of PBS.



Cover tissue sections with the 200µl of Proteinase K solution.

- *General considerations:*

CENBIMO can supply Proteinase K prepared at an optimum working concentration, ready to use once mixed with PBS. If this is not required, Proteinase K should be used at a working concentration of 30µg/ml in PBS pH 7.4.

Efficiency of Proteinase K digestion is affected by varying fixation/decalcification methods and may require optimizing. If weak to no signal is achieved with 10 min room temperature incubation we suggest increasing the incubation time and/or temperature to 55°C. If tissue appears to be over-digested with a 10 min room temperature incubation we suggest further diluting the Proteinase K 50% with PBS pH 7.4 or reducing the incubation period to 5mins at room temperature.

1. *Mix each vial of Proteinase K with 200µl of PBS. Use one vial for one slide.*
2. *Dry around the edges of the slides with tissue paper taking the precaution not to touch the section.*
3. *Cover tissue sections with the 200µl of Proteinase K solution and incubate in a humid chamber for exactly 10 minutes at room temperature.*
4. *Wash well with distilled water. It is not necessary to use an enzyme inhibitor.*
5. *Transfer to PBS pH 7.4 and leave to stand for at least 2 minutes.*



If the slides have been heat treated previously only use Proteinase K for 5 minutes as the process will have significantly sensitized the tissue sections.

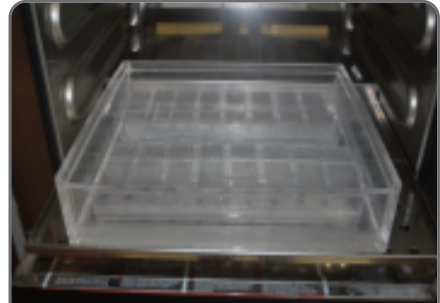
For bone marrows continue to use 10mins after heat treatment. Bone marrows fixed in formic acid or EDTA normally require a Proteinase K digestion of 20min at 55°C. It is important to remove all Proteinase K residues before continuing with the protocol.

## Incubation with the probe

Hybridization. This is the most important phase of the protocol.



Add the 65µl of probe to the tissue section ensuring the entire section is completely covered and avoiding air bubbles.



Place the slides in a horizontal position in a humid chamber and close well.

- *General considerations:*

Any recipient that contains water and can be closed to avoid evaporation may be used as a humid chamber. 62°C is a relatively high temperature and if the chamber is not well closed there will not be sufficient humidity to prevent the probe solution from drying.



**It is important that the temperature of the incubator is a reliable 62°C.**

1. Dry around the edges of the slides with tissue paper taking the precaution not to touch the section and to always leave it wet.
2. Add the 65µl of probe to the tissue section ensuring the entire section is completely covered and avoiding air bubbles. This is best achieved by carefully using a pipette tip to aid spreading of the probe solution.
3. Place the slides in a horizontal position in a humid chamber and close well.
4. **Incubate at 62°C for 1hr.**
5. If no suitable humid chamber is available drop the 65µl of probe over a 24 X 50 mm coverslip and place over the tissue section. Seal the edges of the coverslip with rubber cement. Incubate in a 62°C incubator or over a hot plate for 1 hour. After incubation carefully remove the rubber cement and coverslip and continue the protocol.

Note: It is not necessary to use any type of cover slip.

Both incubator and humid chamber must be at 62°C when incubating the probe.

## Washing the probe

1. Using a Pasteur pipette, wash the surface of the section vigorously with PBS pH 7.4 several times to remove the probe, taking care not to damage or remove the tissue section.
2. Agitate the slides in PBS pH 7.4 for 5 minutes, either manually or using any kind of agitator.

Note: It is not necessary to use any special type of wash or formamide.

## Revealing the probe (Digoxigenin labeled probes)

In this phase the Digoxigenin label in the probe DNA is visualized. This can be performed manually or using automatic apparatus.

- *General considerations:*

As the primary antibody, any commercial anti-digoxin or anti-digoxigenin is appropriate in a suitable dilution as indicated by the manufacturer. CENBIMO can supply primary antibody prepared at an optimum working dilution, lyophilized and ready to use once reconstituted with distilled water. The antibody from CENBIMO contains sodium azide as a preservative and once reconstituted can be stored at 4°C.

As the secondary antibody, any commercial peroxidase-labeled anti-mouse polymers may be used following the protocol provided with the commercial kit.

To reveal the probe use a commercial Diaminobenzidine (DAB) preparation.

Protocol for manual revealing of the probe

1. Reconstitute Anti-Digoxin from Cenbimo with 1ml of distilled water.  
Each vial is valid for 10 slides.
2. Remove excess buffer from sections as before.
3. Cover sections with 100µl of Anti-Digoxin and incubate in a humid chamber for 30mins at room temp.
4. Wash vigorously with PBS, pH 7.4 agitate in PBS for 1min.
5. Remove excess buffer from sections.
6. Drop commercial polymer anti-mouse HRP over the sections (enough to cover the tissue 50-100µl) incubate in a humid chamber following the manufacturer's instructions.
7. Wash vigorously with PBS pH 7.4, agitate in PBS 1min.
8. Remove excess buffer from sections and apply commercial Diaminobenzidine (DAB) following the manufacturer's instructions.
9. Wash with water.
10. Counter stain the sections **briefly (2-3secs)** with Harris hematoxylin **diluted 50%** in water.
11. Wash with water, dehydrate and cover slip following normal laboratory protocols.



## Revealing the probe (DUAL probes)

In this phase the Digoxigenin / Biotin labels in the probe DNA is visualized. This can be performed manually or using automatic apparatus.



As the primary antibodies, any commercial anti-digoxin or anti-digoxigenin (mouse) and anti biotin (rabbit) are appropriate in a suitable dilution as indicated by the manufacturer. CENBIMO can supply primary antibody Anti-Digoxin prepared at an optimum working dilution, lyophilized and ready to use once reconstituted with distilled water. The antibody from CENBIMO contains sodium azide as a preservative and once reconstituted can be stored at 4°C for a period of 1 month.

As the secondary antibody use a cocktail polymer of Anti Mouse HRP + Anti Rabbit AP following the protocol provided with the commercial kit.

To reveal the probes use a commercial Diaminobenzidine (DAB) preparation followed by a commercial Fast Red preparation.



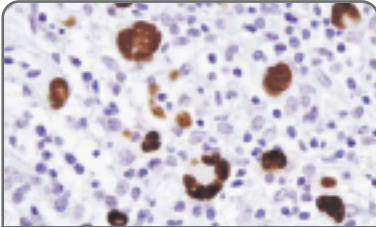
After incubation with the secondary cocktail polymer buffer should be changed to TBS pH 7.4 to avoid saturation of the alkaline phosphatase enzyme by PBS.

1. Reconstitute Anti-Digoxin from Cenbimo with 1ml of high quality distilled water. Each vial is valid for 10 slides.
2. Dilute the Anti-Biotin to working concentration (following the manufacturer's instructions) with the Anti-Digoxin from Cenbimo.
3. Cover slides with 100ul of the resulting primary antibody mix and incubate in a humid chamber at room temperature for 30mins.
4. Wash the slides with PBS pH 7.4.
5. Cover the slides (1-2drops) with secondary anti mouse HRP and anti rabbit AP cocktail polymer and incubate in a humid chamber following the manufacturer's instructions.
6. Wash slides with **TBS pH 7.4** 
7. Make up DAB solution following the manufacturers instructions (normally 1µl DAB in 50µl substrate for each slide)
8. Apply DAB solution (50µl) and observe development by light microscope.
9. Wash with **TBS pH 7.4**
10. Make up Fast Red solution following the manufacturer's instructions (normally 1.5µl Fast Red in 50µl substrate for each slide)
11. Apply Fast Red solution (50µl) and observe development by light microscope.
12. Wash with water and proceed rapidly with counterstain (Harris hematoxylin at **50% dilution**  in water for **3 dips**), dehydration and coverslipping following normal laboratory protocols.

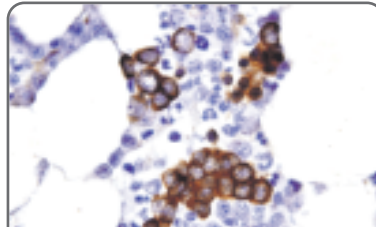
## Interpretation of Results

### HistoSonda General Interpretation

Samples in which HistoSonda expression is observed will show a brownish color in the cell nucleus, cytoplasm or both (depending on the probe), which will contrast over the blue-violet background given by hematoxylin staining



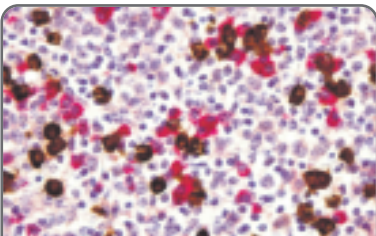
HISTOSONDA® Eber  
Lymph node, Hodgkin's Disease,  
mixed cellularity



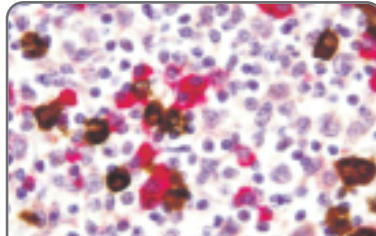
HISTOSONDA® Hemoglobin AC  
Bone Marrow, Myelodysplastic  
syndrome

### HistoSonda Dual Kappa-Lambda

Samples in which immunoglobulin light chains expression is observed will show color in the cell cytoplasm, which will contrast over the blue-violet background given by hematoxylin staining. The color will be brown in the case of Lambda light chain, the color for Kappa light chains depends on the choice of chromogenic reagent used (generally red or blue).



HISTOSONDA® Kappa-Lambda  
Reactive lymph node



HISTOSONDA® Kappa-Lambda  
Reactive lymph node

## HistoSonda c-erbB2

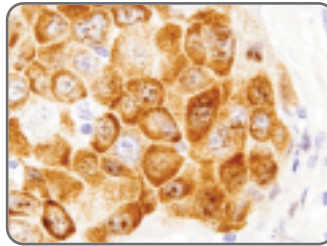
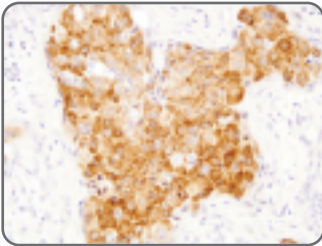
HistoSonda c-erbB2 is a yes or no result. There are no grading systems and no need to count percentages. HistoSonda c-erbB2 targets c-erbB2 messenger RNA and is therefore cytoplasmic staining. There is no membrane staining.

If cytoplasmic staining of any intensity is observed the result should be considered as positive. Intensity of stain does not correspond to a grading system. Absence of cytoplasmic staining should be considered as negative.

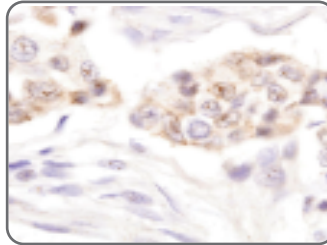
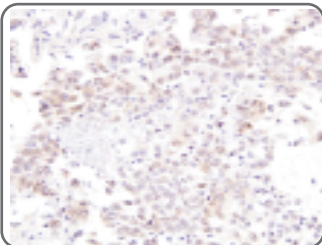
This method has been tested in more than 100 cases and HistoSonda staining has been found to have a high correlation (>98%) with genetic amplification identified by FISH regardless of intensity of staining.

In some sections (both positive and negative) you may observe nuclear dots. This is a phenomenon that does not have any bearing on the results. If nuclear dots are observed without any cytoplasmic staining this is still considered a negative result.

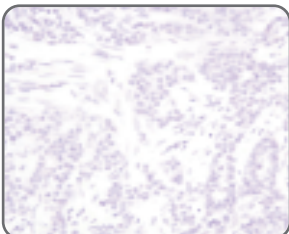
Below are some examples.



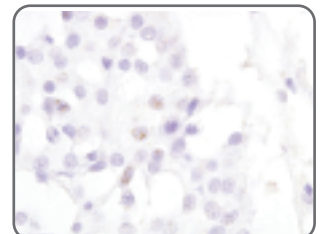
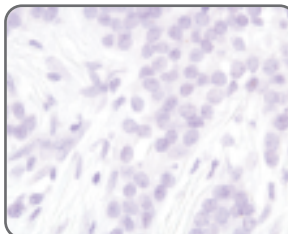
*Strong HistoSonda staining: positive*



*Weak HistoSonda staining: positive*



*Negative HistoSonda staining:  
no nuclear dots*



*negative HistoSonda staining:  
nuclear dots*

## Frequently Asked Questions (FAQs)

### **Can I use my own revealing kit?**

Yes. The choice of chromogenic, peroxidase or alkaline phosphatase labeled revealing system is left completely open to the user.

### **Can I use an automatic immunohistochemistry apparatus to reveal the probe?**

Yes. Refer to the guidance notes in the protocol on which antibodies are suitable and follow the manufacturer's instructions for the apparatus.

### **Can I use PBS with Tween?**

It is not recommended as tween appears to provoke background problems. The only recommended buffers are PBS or TBS at pH 7.4 without tween.

### **Why should the slides not be covered during hybridization?**

Firstly it is simply not necessary to cover the slides when using a humid chamber for the incubation process. Secondly, using a cover slip will cause some of the hybridization solution to be expelled and potential damage to the tissue section. If a cover slip is used the edges must be sealed during the incubation with a rubber cement.

### **How is the probe incubation period only 1 hour?**

The elevated thermal energy generated at 62°C allows molecules to vibrate quicker, therefore taking less time to find their target even though they are situated in a very viscous buffer.

### **Why is the hybridization temperature 62°C?**

62°C is a temperature that permits both rapidity and specificity. The elevated thermal energy allows rapid movement and creates an environment of high stringency. In a high stringency environment short sequences (such as oligos) and longer sequences of low sequence homology only create a few hydrogen bonds when they try to anneal. These few bonds are not sufficient to avoid the separating forces and therefore unspecific annealing is eliminated. Only long sequences of practically 100% homology (the HistoSondas) will be able to anneal due to the stabilizing forces created by the many hydrogen bonds they form with their target sequence.

### **Why is it necessary to use a humid chamber?**

A humid chamber is necessary to prevent drying of the probe solution on the tissue sections. Drying of the solution will produce intense background making identification of positively labeled cells impossible.

### **Can I use less probe?**

The probes are provided in single use form. The use of less than the full amount (65µl) will result in weak labeling and is **NOT** recommended.



### **Can I use tissue sections fixed with B5?**

Yes but the mercury **MUST** be removed after de-waxing using Lugol's solution. If the mercury is not fully removed it will interfere with the binding of the probe with its target.



### **What is the decalcification method recommended by CENBIMO?**

Our recommendation is as follows:

For 1 litre of Decalcifying Solution:

100ml Commercial Formaldehyde (37 - 40%)

9g NaCl

50ml glacial CH<sub>3</sub>COOH (Acetic Acid)

Fill until the 1 litre mark with distilled H<sub>2</sub>O

Depending on the size of the cylinder, decalcify for between 24 and 48 hours. This method both decalcifies and fixes tissue samples.

### **What is the fixation method recommended by CENBIMO?**

100ml Commercial Formaldehyde (37-40%)

900ml saline solution (NaCl 9g/1000ml)

Fix for between 16-24hrs optimum.

### **How thick should I cut my tissue sections?**

For optimal results we recommend tissues should be cut at 4µm. Sections that are thinner than 4µm have greatly reduced quantities of target RNA which will reduce the signal of the results.

### **How recently should my sections be cut?**

Tissue sections should not be more than 1 month old maximum, ideally not more than 2 weeks old. For best results it is recommended to perform the protocol as soon as possible after cutting.



To follow this manual or find out more specific details you can find and watch the Video Demonstration HistoSonda® Protocol at:

<http://www.cenbimo.com/e-learning.html>

**if you need technical help, please contact us at:**  
**[techserv@cenbimo.com](mailto:techserv@cenbimo.com)**

## Troubleshooting

Listed here are some common reasons for unexpected results but if these do not satisfy your query, CENBIMO has a technical department to answer any further questions: Please contact this department at [techserv@cenbimo.com](mailto:techserv@cenbimo.com)

### Weak or no labeling observed

Protocol may have not been followed correctly. Check for omission of steps.

Tissue is negative for this probe.

Insufficient Proteinase K digestion. Check the fixation/decalcification protocol of the tissue sections and increase time and/or temperature (55°C) of the Proteinase K incubation.

Sections have been cut for too long. We recommend that sections are not more than 1 month old. Try cutting fresh sections.

Tissue is producing target mRNA in small quantities.

### Background is observed

Insufficient washing of probe or antibody solutions.

Heat Treatment – If the slides have been heat treated it is possible that the duration was too long. Decrease the time in the microwave slightly and remember to remove the slides and transfer them to room temperature distilled water immediately.

Deproteinization – Proteinase K incubation period too long or Proteinase K not sufficiently removed. Tissue may have been damaged. Proteinase K should be removed by vigorously washing in distilled water immediately, changing the water several times.

Incubations – Probe or other incubation solution may have dried to tissue section. Use a humid chamber for all incubations making sure that it is well closed. Check slides after each incubation to be sure that solutions have not dried, and wash tissue sections well.

### Tissue has lost morphology

Proteinase K over-digestion. Decrease the incubation period and temperature (room temperature). If overdigestion still occurs dilute Proteinase K in PBS pH 7.4.

Physical damage due to cover slipping during probe incubation. Where possible always omit the use of cover slips for probe incubation and use a humid chamber.

### A cloudy precipitate remains in the HistoSonda tube after reconstituting.

Pipette up and down to break up the precipitate. Vortex the tube and centrifuge for 10 seconds. If unusually, the precipitate still remains, you can cover the tissue section with the entire solution including the precipitate, using a pipette tip to spread evenly. This will not adversely affect the hybridization process or results. After hybridization, wash vigorously in PBS to remove any trace of precipitate. Alternatively, the precipitate may be dissolved as stated in the protocol, by introducing the tube in an incubator at 62°C for 15mins prior to use.

### Eosinophils have been labeled positive

Heat treatment of tissue sections may either have been omitted or insufficient. Increase the time in the microwave slightly and remember not to remove slides until you see uniform boiling (bubbles rising from the bottom of the wash bath), however also take into account that increasing the time more than necessary will result in background labeling.

### Unexpected results when using Bone Marrow sections

The variable nature of bone marrow processing can affect the deproteinization efficiency of Proteinase K. If you observe weak or no labeling when using bone marrow sections we advise to increase the time and temperature (55°C) of deproteinization until suitable results are obtained.

# HistoSonda

[www.histosonda.com](http://www.histosonda.com)



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