Dako

Agilent Pathology Solutions

Troubleshooting Guide for RNA CISH Protocols

This guide describes the function of each step in the RNA CISH protocol as well as whether the step is modifiable and the effect modification has on assay performance.

Step 1: Two Phase Dewax Wash ISH

In this step the paraffin surrounding the tissue is removed by Clearify and then washed with Pretreatment buffer.
This step is not modifiable.

Step 2: Target Retrieval ISH

Pretreatment buffer and heat are used to reverse crosslinking caused during the fixation processes. For DNA ISH, a temperature of >95°C is needed. For RNA ISH, a lower temperature is used. 70°C was empirically determined to be optimal. Lower temperatures may lead to higher background or lower signal. Higher temperatures may result in loss of tissue morphology.

During development, combinations of pretreatment temperature and pepsin digestion time were evaluated: high temperature (97°C) pretreat and short pepsin (3 minutes) or low temperature pretreat (70°C) and longer pepsin (15 minutes). We found that lower pretreat/longer pepsin gave better tissue morphology – especially on bone marrow samples where we were seeing loss of tissue using a 97°C pretreatment. High temperature and long pepsin destroys the tissue morphology.

Step 3: Enzyme Pretreatment

Step 3a: Ethanol wash removes residual reagents.

This step is not modifiable.

Step 3b: Pepsin is used to digest cellular proteins to allow the probe access to the target RNA.

15 minutes was chosen for optimal signal and tissue morphology. Shorter times may result in lower signal, and longer times can negatively affect morphology and/or adhesion of the sample to the slide.

Step 4: Hybridization

The slide is dried, probe is applied, and covered with ISH lid.

Denaturation is performed at 66°C for 10 minutes to remove any secondary structure in the RNA. The temperature is not modifiable. Time can be changed but has little effect on assay performance.

Hybridization is performed at 45°C for 1 hour to allow probe to bind RNA target. The temperature is not modifiable. Time can be increased up to 2 hours. Little difference in performance was observed when hybridization time was extended, but it is possible that longer times will increase hybridization in some samples.

Step 5: Stringent Washing

Stringent wash removes un-bound probe to give clean background. Washing for 3 minutes at 55°C results in optimal signal to noise. Higher temperature or longer times resulted in loss of a signal. Lower temperatures gave unacceptable background.

Depending on sample type, the user could modify this step to give optimal results.

Step 6: Endogenous Enzyme Block

The sample is treated with reagent to suppress endogenous enzyme activity. This is a required step and modifying the time has little to no effect on the result.

Step 7: Primary Antibody

The sample is incubated with Anti-FITC-AP to bind the FITC-label on the probes. Incubating for 30 minutes is sufficient for most tissues. Longer times may result in more binding, shorter times in less binding.

Step 8: Substrate Chromogen

BCIP/NBT is added to sample and incubated to allow reaction by the bound Anti-FITC-AP to form the dark blue/purple precipitate. Two rounds of 20 minutes each are performed to maximize signal. Reducing either the time or number of applications will lower the intensity of the signal.

Step 9: Counterstain

Nuclear Fast Red (NFR) is applied to stain the nuclei pink. 10 minutes gives a medium pink color on most tissues. Longer times will give darker pink, shorter times, lighter pink. This step can be modified to meet the user needs with respect to contrast.

Step 10: Unload Slides from Omnis

The slides will be dry when then are unloaded from the Omnis. Slides should be dehydrated with alcohol, cleared with xylene and mounted with a non-aqueous mounting medium.

Possible suboptimal results and suggested protocol modifications:

1) Signal is low or non-uniform

1a) Make sure probe mixing device is working properly and probe is thoroughly thawed and mixed.

1b) Reduce the stringent wash temp to 50°C.

1c) Check that the instrument is level.

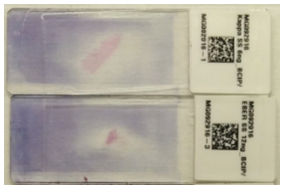
1d) Check that the hot plate is clean.

2) Staining is dark but background is seen either in patches or across the tissue*

2a) Increase stringent wash temperature in 3°C intervals to determine if background can be removed while maintaining specific staining.

2b) Reduce the Substrate Chromogen incubation cycle to 1x and/or reduce the time.

2c) Inspect BCIP-NBT Substrate (Dako Omnis) vial. If contents are dark blue/purple and a precipitate has formed at the bottom of the vial, replace with a new vial.



*If using Dako FLEX slides, some blue staining will be observed on the slide outside the sample area. This does not affect the scorability of the sample, but if it is a problem, Super Frost + slides may be used to eliminate this effect. Example of slide background.

3) Tissue morphology is suboptimal or tissue is lost during processing

3a) Ensure positively charged slides are used.

3b) Reduce the pepsin incubation time in 5-minute intervals.

Protocol steps and allowable time and temperatures:

Phase	Step	Reagent & Volume	Default	Time & Temp Customization
Dewax	Dewax	Clearify Clearing Agent	10 min.	On/Off
		(Dako Omnis), 95 mL	38°C	Separate CISH template for no Dewax
		Rinse with 250 mL of ISH Pretreatment Solution	65°C	
Pretreatment	Target Retrieval	ISH Pretreatment Solution (Dako Omnis), 125 mL	3 min.	3-60 min.
		Cooling Solution 180 mL ISH Pretreatment Solution	70°C	60-97°C
Pepsin Digestion	Wash	Di Water	3 min.	N/A
		96% EtOH (Dako Omnis) 300 µL	3 min.	
		96% EtOH (Dako Omnis) 300 µL	3 min.	
		Di Water	2 min., 32°C	
	Digestion	ISH Pepsin (Dako Omnis), 300 µL per slide	15 min.	3-60 min.
		Di Water	2 min., 32°C	32°C
	Drying	Di Water	15 min.	N/A
		Drying	45°C	
Denaturation & Hybridization	Denaturation	Fluorescently-labeled RNA CISH probe	10 min., 66°C	5-20 min., 66°C
	Hybridization		60 min., 45°C	30-120 min., 45°C
Stringent Wash	Stringent Wash	ISH Stringent Wash Buffer (Dako Omnis), 125 mL	3 min.	3-20 min.
		Cool Down ISH Stringent Wash Buffer 250 mL	55°C	50-75°C
CISH staining	Enzyme Block	Wash Buffer	3 min.	3-60 min.
		CISH Endogenous Enzyme Block (Dako Omnis)	3 min.	
		Wash Buffer	2 min., 32°C	32°C
	Primary Antibody	Anti-FITC-AP (Dako Omnis)	30 min.	4 – 60 min.
		Wash Buffer	2 min., 32°C	32°C
	Substrate Chromogen	BCIP-NBT Substrate (Dako Omnis)	20 min.	3 - 20 min.
		Wash Buffer	2 min.	
		BCIP-NBT Substrate (Dako Omnis)	20 min.	3 - 20 min.
		Wash Buffer	2 min.	
		DI Water	30 sec.	
		Wash Buffer	2 min., 32°C	
Counterstaining	Counterstain	Nuclear Fast Red (Dako Omnis)	10 min.	3 – 60 min.
		Di Water	4 min., 32°C	
Dry and Unload	Dry		5 min., 45°C	5-30 min., 45°C

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