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Quality Control Considerations for Fluorescence *In Situ* Hybridisation of Paraffin-Embedded Pathology Specimens in a Diagnostic Laboratory Environment

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Additional information is available at the end of the chapter

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1. Introduction

Paraffin FISH testing is the application of the fluorescence *in situ* hybridisation (FISH) methodology to formalin fixed paraffin embedded sections (FFPE), and has proven a powerful tool for both histopathologists and cytogeneticists. Pathologists use the method to confirm or exclude a histological diagnosis, to differentiate between tumour subtypes, or as a confirmatory tool where the tissue morphology is poor or the immunohistochemistry (IHC) staining is uninformative [1]. Similarly, cytogeneticists find it useful when the tissue sample is insufficient or unsatisfactory for conventional culture methods, or when such methods fail to yield a result. The method can also be used to confirm abnormalities found in other tissue samples. Paraffin testing has a further advantage over conventional cytogenetic and molecular testing methods, as it can localize the anomaly within specific cells or tissue areas, and this provides the ability to study anomalies at a single cell level [2,3], unlike DNA techniques that pool DNA from hundreds of different cells [1,3].

Compared to FISH testing on conventional suspension samples (Figure 1), paraffin FISH can be labour intensive and highly variable due to differing fixation times between samples and referring histology labs, and the interpretation may be limited due to truncation of signal and overlapping cells [1,4].

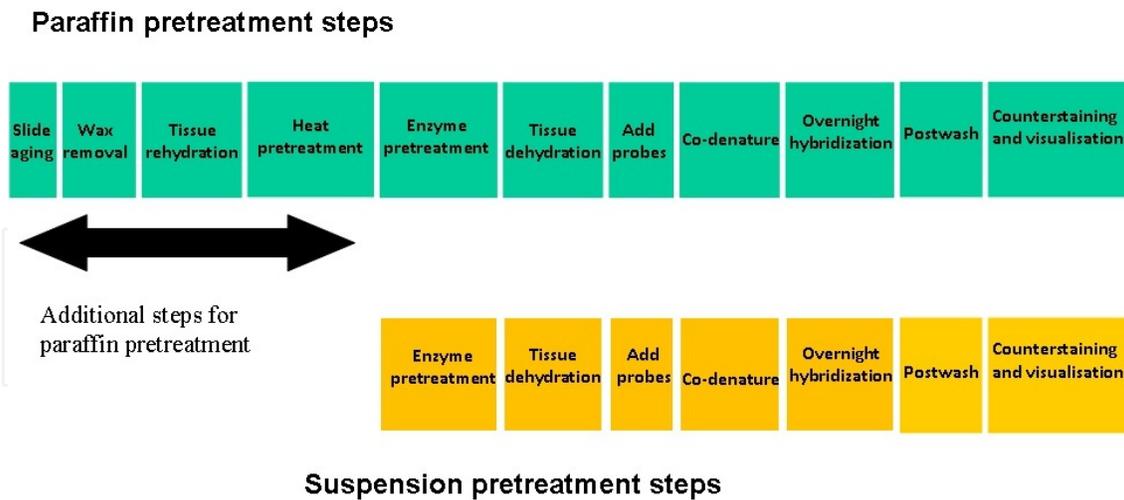


Figure 1. A comparison of the paraffin pre-treatment process with the conventional FISH pre-treatment process on suspension samples.

For these reasons, it must be considered separately from the conventional suspension FISH method, and while it can be used as either a stand-alone technique, or an adjunct to conventional cytogenetics techniques [5], it must be noted that due to the use of interphase nuclei, a prior knowledge of the anomaly of interest is required.

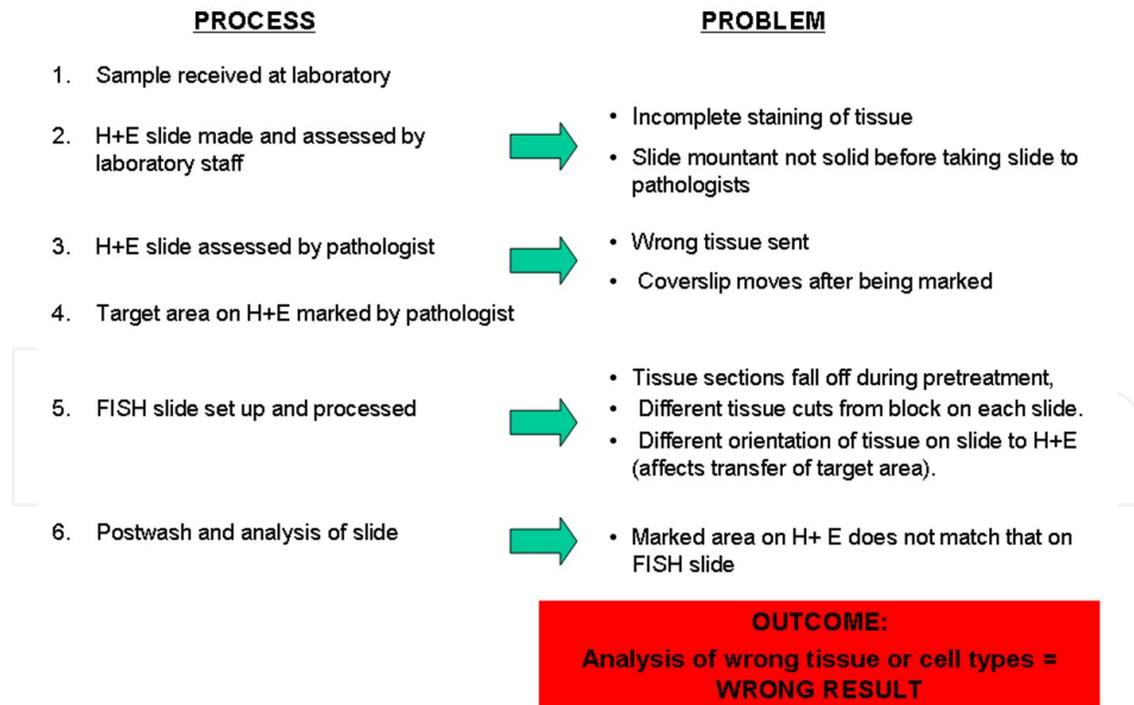


Figure 2. Errors that occur during the paraffin pre-treatment process.

The basic premise of the method involves establishing the area of interest for testing on the H+E stained pathology slide, and transferring this area to an unstained paraffin slide, which is then

pretreated, probed and co-denatured using the traditional FISH methodology [6,7]. However, one of the most crucial factors for paraffin analysis is the assessment of the correct target area before beginning the procedure – without this, an erroneous result may occur (Figure 2), which may be costly to patients if it results in the appropriate treatment being withheld [1].

For this reason, robust internal and external quality control procedures are required for diagnostic paraffin FISH testing and the exclusion of non-target tissue before analysis decreases the likelihood of an incorrect result due to an analysis error [1]. This protocol therefore aims to provide a guide to some of the considerations and troubleshooting that are necessary when using the method for diagnostic medical testing. It is adapted from the method used by the Diagnostic Genetics Department, LabPlus at Auckland City Hospital, New Zealand. There are a number of variations to the basic FISH method that can be used depending on the nature and number of samples being processed, and new technology has also been developed to automate the process (Xmatrix, Abbott Molecular). In this protocol however, we have suggested extra steps that are designed to help improve the quality of the testing procedure for diagnostic use. Probes used for diagnostic testing are commercially available and may be downloaded and gathered from the websites of companies such as Abbott Molecular, Cytocell, Zytovision or Kreotech Diagnostics.

2. Method

One slide (2-5 micron thickness usually) is needed per probe or probe set, and if a haematoxylin and eosin (H+E) slide is not provided by pathologists, an extra slide must also go through the deparaffinisation steps before staining with the Shandon Rapid-Chrome™ Frozen Section Staining kit (alternatively the individual stain kit components can be made from powder).

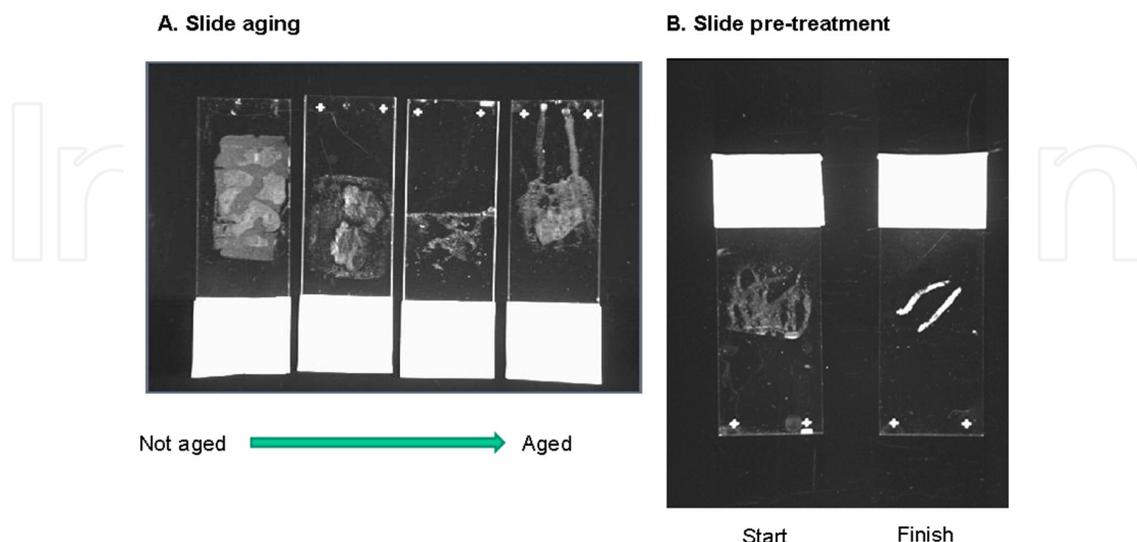


Figure 3. Slide pretreatment steps for paraffin FISH. (A) Appearance of unstained paraffin slides after aging in a 60°C oven - note melted or "bubbled" appearance. (B) Unstained paraffin slides and after the pre-treatment steps.

1. Deparaffinisation (approx. 60 minutes); see Figure 3
 - a. Leave slide/s on the hotplate/in the oven at approximately 65°C for 30-60 minutes for aging (Figure 3).
 - b. Perform deparaffinization by placing slide/s in xylene for at least 10 minutes in the fume hood, with intermittent shaking.
 - c. Rehydrate slide/s by placing them for 2 minutes in each of 100%, 80%, and 70% ethanol solutions, followed by deionised water at room temperature.
2. Haematoxylin and Eosin (H+E) slides; see Figure 4

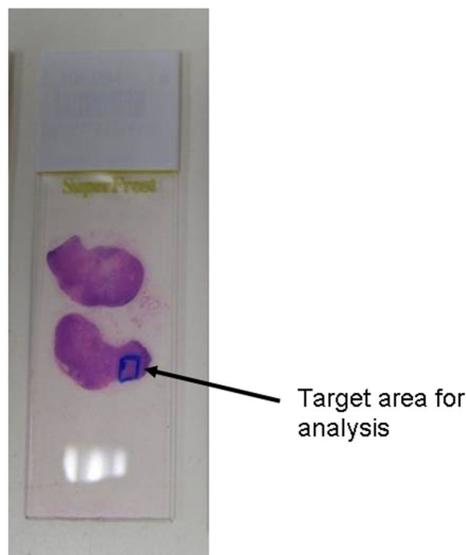


Figure 4. A haematoxylin and eosin (H+E) stained slide with the target area for analysis marked by a pathologist.

- a. Take rehydrated slide/s and stain using the Shandon Rapid-Chrome™ Frozen Section Staining kit and mount the slide using Shandon Mount.
 - b. Leave slides on the hotplate for at least 30 min to dry the mountant.
 - c. Check slides for stain quality under a light microscope.
 - d. Take slide/s to pathologist for marking (Figure 5).
3. Heat Pre-treatment (approx. 30 minutes)
 - a. Add 35µl of heat pre-treatment solution (Invitrogen Tissue Pre-treatment Kit) to the slide/s, cover with a 22x22mm (or bigger sized cover slip) glass cover slip and seal with rubber cement. Alternatively slides can be heat-pre-treated in coplin Jar at 95°C or pressure cooker.
 - b. Heat slide/s on the thermal cycler for 15-60 minutes at 95°C (The time is dependent on the type of tissues and length of formalin fixation).

- c. On completion, immerse slide/s with cover slip in deionised water to cool down and gently remove the cover slip.
 - d. Wash briefly in a coplin jar of deionised water at room temperature and drain off excessive water.
4. Enzyme Digestion (approx. 40 minutes).
- a. Add an appropriate amount (~15µl) of enzyme reagent (Invitrogen Tissue Pre-treatment Kit) to the slide/s, depending on the size of hybridisation area, and cover with a square of parafilm.
 - b. Incubate slide/s for 15-45 minutes in a humidified chamber at 37°C (This time is dependent on the type of tumours and length of formalin fixation).
 - c. Remove cover slip/s and wash briefly in a coplin jar of deionised water at room temperature.
 - d. Dehydrate slide/s for 2 minutes each in each of 70%, 80% and 100% ethanol solutions and air dry at room temperature. Please note that a different ethanol series is used for the dehydration steps to avoid reagent contamination issues.
 - e. Check the tissue morphology of the pre-treated slide looks the same as that of the H+E.
 - f. The pre-treated paraffin slide/s should then be carefully matched against the marked H&E slide/s, and the area for testing transferred to the pre-treated slide/s using a marker pen initially, followed by the diamond-tipped engraver. This means that the area can still be visualised after the post-wash steps.

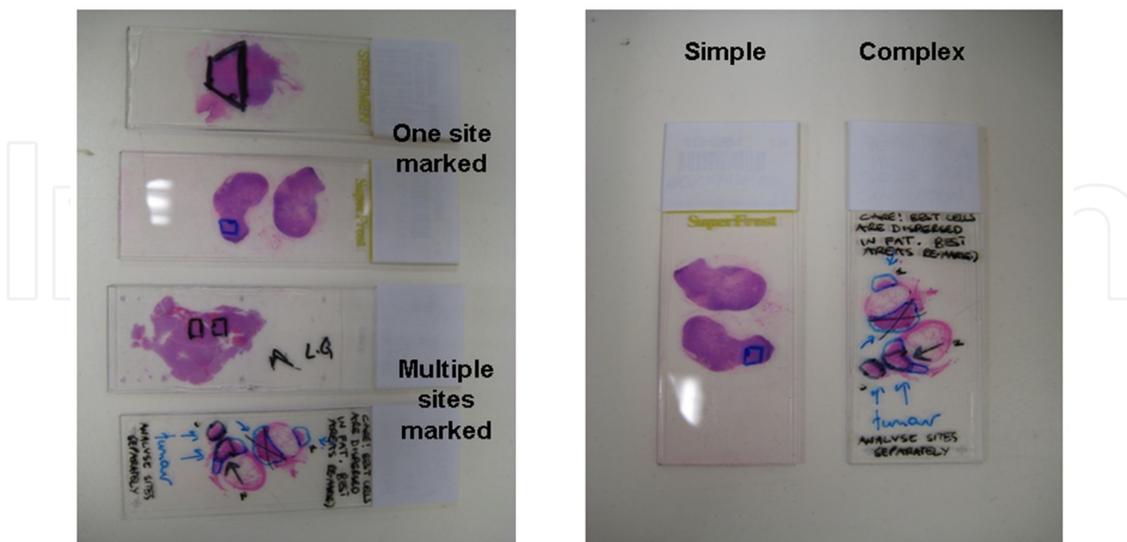


Figure 5. Haematoxylin and eosin (H+E) stained slides marked with the target area for analysis. This reduces the volume of probe necessary and ensures that non-target tissue is excluded as much as possible before the FISH analysis procedure.

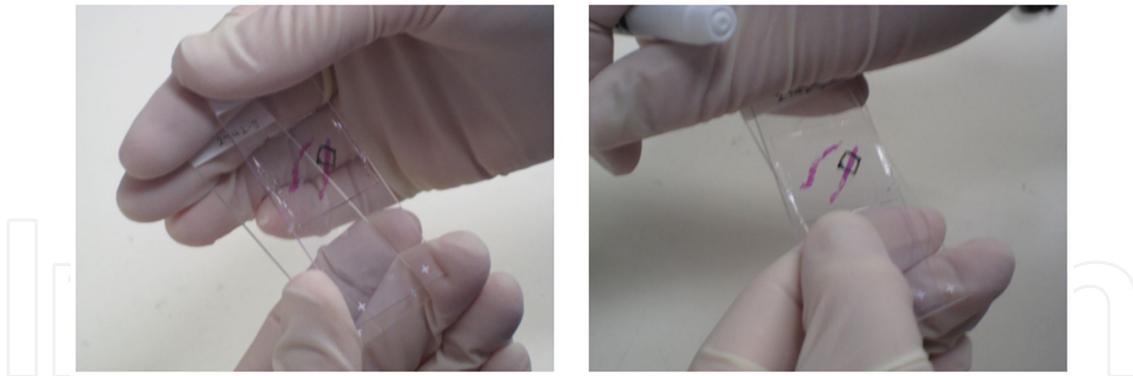


Figure 6. Transfer of target area for analysis from the H+E slide to the pre-treated FISH slide prior to the probing steps.

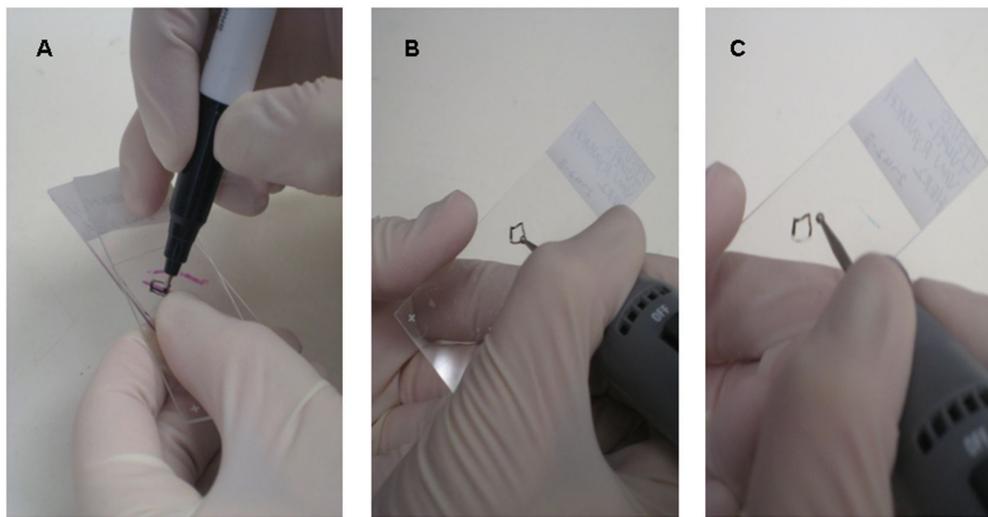


Figure 7. Engraving of target area on to the pre-treated paraffin FISH slide. (A) Draw target area onto bottom of slide with fix-resistant pen. (B & C) Engrave marked area onto bottom of slide using diamond-tipped engraver to keep area visible after post-wash steps.

5. Probe preparation (approx. 10 minutes)

Use Ready-To-Use probes or refer to the probe preparation protocol outlined by the manufacturer.

6. Co-denaturation and hybridization (approx. 25 minutes)

- a.** Apply an appropriate amount (2-10 μ l) of probe mix to the hybridization site marked on each slide, depending on the size of cover slip being used, and seal with rubber cement. Leave the slide/s in the incubator or in a drawer at room temperature for a few minutes to allow the rubber cement to dry before placing them in the thermal cycler.
- b.** Denature slide/s together with probe mix for 10-20 min at 85°C or 5-10min at 95°C.
- c.** After co-denaturation, slide/s may be placed in a humidified box in the incubator at 37°C for at least 12-16 hours, usually no more than 72 hours.

7. Post Hybridization Wash (5 Minutes)

- a. Briefly soak slide/s in 2xSSC and gently remove rubber cement.
- b. Wash slide/s in 0.4xSSC/0.03% Tween 20 (or NP40) at 72°C for 2 min.
- c. Place slide/s in 2xSSC/0.01% Tween 20 (or NP40) for 1 min.
- d. Briefly drain slide/s, apply DAPI counter stain and put cover slip on.
- e. Visualize FISHed-slide/s under fluorescence microscope.

When using indirectly labelled commercial probes that require antibody detection, signal detection must be done according to the manufacturer's instructions.

8. Analysis and interpretation; see Figure 8.

- a. With a pathologist's consultation, check the H+E slide on a transmitted light microscope to assess whether the sample contains a mixture of cell types, as this may affect the interpretation of the FISH signal pattern.
- b. Check the paraffin FISH slide on a fluorescence microscope using the 10x objective to ensure the area marked on the slide approximately matches that on the H+E slide.
- c. Using two observers, analyse a minimum of at least 8 representative sites within the marked region (a minimum of 4 different areas per observer), scoring only cells that show both the target and control loci. Analysis of areas of areas where the cells are not overlapped is preferable, and a third analyst is required where there is discordance between two observers.



Each analyst must:

1. Check histology slide for mixed cell population
2. Check paraffin FISH slide to ensure that area matches
3. Score areas with minimal overlap
4. Observers score a minimum of 8 representative sites in marked area (different QAP programmes may use fewer number of sites).

Figure 8. Analysis principles for paraffin FISH slides.

3. Troubleshooting

Problem: Unclear whether slides have been aged before arrival, as repeating this step may decrease the hybridization efficiency of the probe.

[Step 1]

Solution: Although some waxes do not change in appearance, pre-aged slides generally have a bubbled or melted appearance of the wax compared to the smooth appearance of non-aged slides in general (N.B: some wax types do not change in appearance so this is a rule of thumb only).

Problem: The use of xylene to remove the wax from around the sample is not ideal as xylol is highly toxic.

[Step 1]

Solution: An alternative to xylene is HemoDe from Scientific Safety Solvents.

Problem: Finding that the wrong tissue was sent by the referring laboratory.

[Step 2]

Solution: Ask for a copy of the pathology report to be sent with all samples, and get pathologists to ring the referring laboratory to request the appropriate sample for testing.

Problem: Incomplete staining of the H+E slide causing correct target area to be missed by pathologist.

[Step 2]

Solution: Slides should be quality checked before taking them to a pathologist. Check the stain by eye to see if there are obvious colour differences across the slide – if one of the stains has been missed in an area it will appear either a dull purple (eosin missed) or a dull pink (haematoxylin missed or there is a problem with the pH of the bluing reagent) compared to the rest of slide. If there are any doubts, ask a histopathology technologist for assistance.

Problem: Cover slip moves after the slide has been marked because mountant is not completely hardened. This causes the target area to move.

[Step 2]

Solution: Leave the slides on the hotplate for a longer period of time, or change mountant to a faster drying version such as Entellan (Note: it is not possible to remove the Entellan with methanol after it has been cover slipped, hence why DPX is the preferred mountant).

The Rapid-Chrome™ Frozen Section Staining kit uses Shandon Mount; however alternatives such as Entellan are available.

Problem: Disappearance of tissue on slide during dehydration steps.

[Steps 3 and 4]

Solution: The ethanol series (in step 1) is necessary to rehydrate the tissue for the enzyme solution to act on, and may cause the tissue to become translucent, however it will become white again once the slide is dehydrated.

Problem: Scratching or loss of tissue during washing steps. Small tissue samples (e.g. core biopsies) may become fragile during the pretreatment steps and fall off the slide.

[Steps 3 and 4]

Solution: As the tissue becomes soft during pre-treatment it may easily fall off or get scratched; coplin jars of deionised water can be used to dip slides into rather than the more aggressive use of squirter bottles or running tap water (do not leave the pre treated slide in water for a long time, especially for a core biopsy or a tiny sample). The size of the tissue gives a good indication as to the fragility of the tissue, so this should be taken into account before beginning the pre treatment steps. Increasing the ageing step may also help to fix the tissue to the slide better, although it may also decrease the hybridization efficiency of the probe to the sample. Alternatively, skipping the heat pretreatment step and doing a reduced enzyme treatment on the sample may combat this.

Problem: The tissue does not look the same as the H+E slide after dehydration steps.

[Step 4]

Solution: This can either be due to loss of tissue during pretreatment or different cuts through the tissue block. Untreated slides should be closely examined to find one that appears to match the pretreated slide and a new H+E slide created using this slide. See also steps for reducing the loss of tissue during pretreatment.

Problem: Transfer of area is difficult due to a slight difference in the morphology of the tissue in different layers of the tissue section, or different orientation of tissue on pre-treated slide to that of the H+E slide.

[Step 4]

Solution: If the morphology of tissue on the pre-treated slide looks different to that of the H+E slide, check it against the remaining untreated slides to see if it looks like tissue has been lost during the pre-treatment procedure. If tissue has been lost, simply start the procedure over again with a new slide. If the morphology of the tissue appears different between the untreated slides, ask a pathologist for help selecting an appropriate slide to pre-treat, and try to find two similar untreated slides. Pre-treat one and make the other into an H+E slide to allow for more accurate marking.

Problem: There is more than one target area marked on slide – is more probe required?

[Step 4 and 5]

Solution: Assess the size of the areas – if there are several small areas, the total volume of probe does not need to be increased, simply aliquot the volume of probe equally over the different areas and place a small cover slip over each. More than one aliquot of probe is only required if the areas are greater than can be covered by a 13mm diameter cover slip.

Problem: The hybridisation buffer for a probe runs out.

[Step 5]

Solution: As hybridisation buffers are all fairly similar, it is fine to use the buffer of similar probe as a substitution. Alternatively, hybridization mix can be made up:

Hybridization mix

(10% dextran sulphate, 50% formamide in 2xSSC, 0.1% SDS, pH 7.0)

1. Mix 12.5ml formamide, 2.5ml 20xSSC pH7.0 and 10ml MilliQ water. Adjust pH to 7.0 with HCl then transfer to a 50ml Falcon tube.
2. Add 2.5mg dextran sulphate and place on a roller mixer at room temperature for 1-2 hours.
3. Add 25µl Tween 20 and invert to mix.
4. Aliquot 500µl into sterile eppendorf tubes. Store at -20°C and use a fresh aliquot each time.

Problem: A thermal cycler is not available for use.

[Step 6]

Solution: Denaturation of the slide(s) can be done separately using 70% formamide/2xSSC, as it gives better quality denaturation although the downside is that it is highly toxic. The hybridisation steps can also be done adequately in a programmable system (e.g. Thermobyte).

Problem: The cover slip is hard to remove before the post wash steps.

[Step 7]

Solution: Place slide in 2xSSC solution and agitate gently after removing the rubber cement, and then remove cover slip. If the cover slip is still stuck to slide, slide the blade of a scalpel under one corner of the slide and lift gently before immersing the slide in a 2xSSC solution and agitating it gently. This may need to be repeated several times if the cover slip remains stuck.

Problem: Weak or patchy signal quality.

[Step 8]

Solution: This can be difficult to fix, as it primarily occurs as a result of poor handling and fixation of tissue prior to receiving the sample for FISH testing [8,9]. Different tissue samples may require the pretreatment times to be varied [10]. The heat pretreatment buffer prepares the tissue for the enzyme to act on and the enzyme degrades the cellular material away from the DNA, in order to allow the probe to anneal to the chromatin. Variation of either or both these times is effective, and the steps may be repeated on the probed slide to reduce the need for lengthy pretreatment times on a new slide. Bone samples such as trephines may show poor hybridization efficiency of the probe, and require hydrogen chloride treatment, unless the sample has already been decalcified prior to arrival.

Poor signal quality may also be a result of incorrect post wash stringency. There is an alternative wash technique that uses 50% formamide/2xSSC to increase the stringency of the wash. However, this is not always ideal, as it significantly increases the length of the post wash, and also uses formamide which is extremely toxic [11].

Problem: High levels of cross hybridization due to non-specific binding of probes.

[Step 8]

Solution: This is due to incorrect stringency of the post wash [1]. For a quick fix, slides can be rewashed using the quick wash procedure reported here, or alternatively washing at a higher temperature or use of a different post wash procedure can be tried [11].

Problem: Cells only show one signal colour.

[Step 8]

Solution: Only cells showing both the control and target loci should be scored (e.g. 2R2G), so if both the control probe and the probe for the region of interest are on the same chromosome, it is most likely to be due to poor hybridisation of one of the probes. First check to see using single colour filters whether the signal colour is present but weak – if it is, repeat the pre-treatment and hybridisation steps again on the same slide (for a shorter time e.g. 15/15 buffer: enzyme treatment).

Problem: Using an indirectly labeled probe and can't get a good signal quality.

[Step 8]

Solution: In most cases, amplification with only a primary antibody is necessary, and further amplification can also increase the level of background on the slide(s). However if the signal is not bright enough, carefully remove the cover slip, rinse slide in 1xPBS (or SSC) and perform further amplification steps with secondary or tertiary antibodies as many times as necessary. After adding each antibody, slides should be covered with parafilm and incubated in a humidified chamber at 37°C for 5 minutes before being washed in 4xSSC/0.05% Tween20 for 2 minutes. Then mount with 8µl Vectashield antifade solution with DAPI.

Problem: Distinguishing between real signal and background or 'rubbish' on slide.

[Step 8]

Solution: Look at the signal intensity on single colour filters – rubbish generally appears to be brighter and shinier compared to real signals, and background will appear fuzzy and indistinct compared to real signal. High background may be due to the slides not being properly sealed with rubber cement during the pretreatment steps, as this allows the solution to evaporate and the tissue to dry out.

Problem: High background on the slides when analyzing.

[Step 8]

Solution: High background may be due to insufficient removal of material during the pre-treatment steps. With high case numbers, solutions can become contaminated, therefore the

solutions in the pretreatment steps need to be changed regularly, and it pays to have an additional coplin jar of 100% ethanol to dip the slides into after the xylol step in order to reduce contamination from the xylol solution. Alternatively, background may be due to the cover slip not being sealed properly during the pretreatment and co-denaturation steps, causing the tissue to dry out. By placing the slide in the incubator to allow the rubber cement to dry before these steps, this effect can be reduced. The use of a glass coverslip rather than a plastic coverslip also helps, as plastic acts as an insulator, and therefore will hold the temperature and increase the drying of the tissue.

The use of detergents in the post wash steps also helps to solubilize proteins, and if Tween20 is not effective, then NP-40 can also be used.

Problem: There is a mixed cell population in the marked target area (e.g. Tumour cells with non-target lymphocytes also present); see Figure 9.

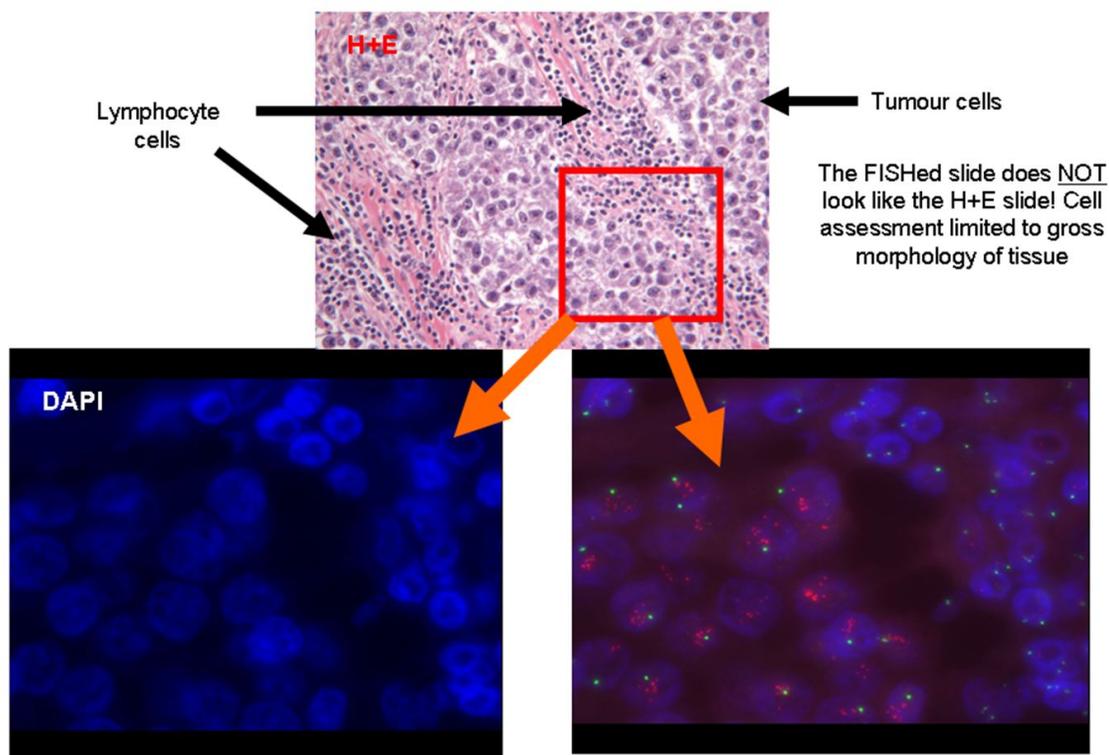


Figure 9. The analysis of slides with mixed tissue populations.

[Step 8]

Solution: Check the H+E slide first before analysing the FISH slide to see whether there is clustering of cell types, or differences in morphology between the different cell types. Then scan the marked target area on the FISH slide using the 10x objective to find areas which appear to be targeted cells and switch to a higher objective for confirmation and then analyse using appropriate filter. Consideration of accidental analysis of non-target cells must also be taken into account when interpreting such cases, therefore increasing the number of cells or sites analysed will increase the accuracy of the analysis. Alternatively, it may be pos-

sible to get a pathologist to mark several smaller sites containing only target cells, as this reduces the risk of error before beginning the analysis.

Problem: Target area marked is very small, so it is difficult to test a variety of areas.

[Step 8]

Solution: While this makes analysis difficult, switching to the 10x objective and moving the stage to a different position will reduce the likelihood of reanalyzing the same cells. Numerical scoring is also preferable in such a case, as it provides a reliable basis for interpretation.

Problem: The cells are highly dispersed or highly clustered, making analysis difficult.

[Step 8]

Solution: Select good areas where the cells are not overlapping using the DAPI filter and use numerical scoring of individual cell signal patterns (this may mean increasing the number of sites examined if the cells are widely dispersed). If a gene rearrangement probe is being used, it may be sufficient just to report the presence or absence of a rearrangement without doing individual cell analysis.

Problem: Distinguishing between real loss and gain of signal compared to artefact.

[Step 8]

Solution: If the target abnormality is either a gain (trisomy/tetrasomy) or loss (deletion) of a signal, it pays to establish thresholds using normal control slides to estimate the level of artefactual gain or loss of signal, and to check the manufacturer's product information to see if splitting of the probe or non-target binding/polymorphisms are common with the probe. The variance in the signal patterns can also be checked – if the percentage of cells showing a 1R2G signal pattern is roughly equivalent to those showing a 2R1G signal, then it is reasonable to assume that it is due to artefactual truncation of signal.

Problem: There is discordance between analysts.

[Step 8]

Solution: Get a third analyst to score the sample. If two analysts have similar results, discard the third analysis, or if all three give different results, take an average of all three results to allow robust interpretation. If the three results differ hugely, it is preferable to confirm the result with a secondary probe where possible, or request a repeat sample from another block. Where the interpretation is still not clear, the case can be reported as inconclusive or failed.

Problem: A low level abnormality, multiple clones or mosaicism is suspected.

[Step 8]

Solution: Where the result is not straightforward use quantitative scoring and use appropriate thresholds for interpretation. Paraffin FISH is not the most suitable method of detection for these cases, although methods that involve taking thicker slices of the section have been developed [12].

4. Conclusion

The role of pathologists is crucial to the analysis of paraffin FISH sections from the beginning of the process. They can help to eliminate very basic laboratory errors, such as identifying whether incorrect tissue has been sent prior to processing the slides, and can also help to identify the appropriate target tissue within the paraffin section prior to analysing the sample, so that inappropriate tissues can be reduced or eliminated. When analysing products of conception, the fetal component can be very small compared to the maternal component, and without guidance of pathologists, an erroneous result may occur. Similarly, in breast cancer samples, it is important to eliminate areas of contained carcinoma (*in situ* components such as DCIS and LCIS) and lymphocytes, as these may result in false positive or negative results, which can be deleterious if treatments such as Herceptin are then withheld from the patient. Some samples such as lymphomas or graft versus host disease may require extensive guidance from pathologists as knowledge of the disease characteristics will allow for highly targeted analysis. In follicular lymphoma, the follicles need to be identified so that centrocytes and centroblasts are targeted for analysis, and normal lymphocytes and reactive cells are avoided when analysing the sample (Swerdlow et al. 2008). For this reason, it is best to include a variety of areas to get a representative result. It should be noted that external quality assurance programmes may differ in the number of sites required for analysis. Generally speaking, fewer sites are required, if initially the non-target tissue is eliminated.

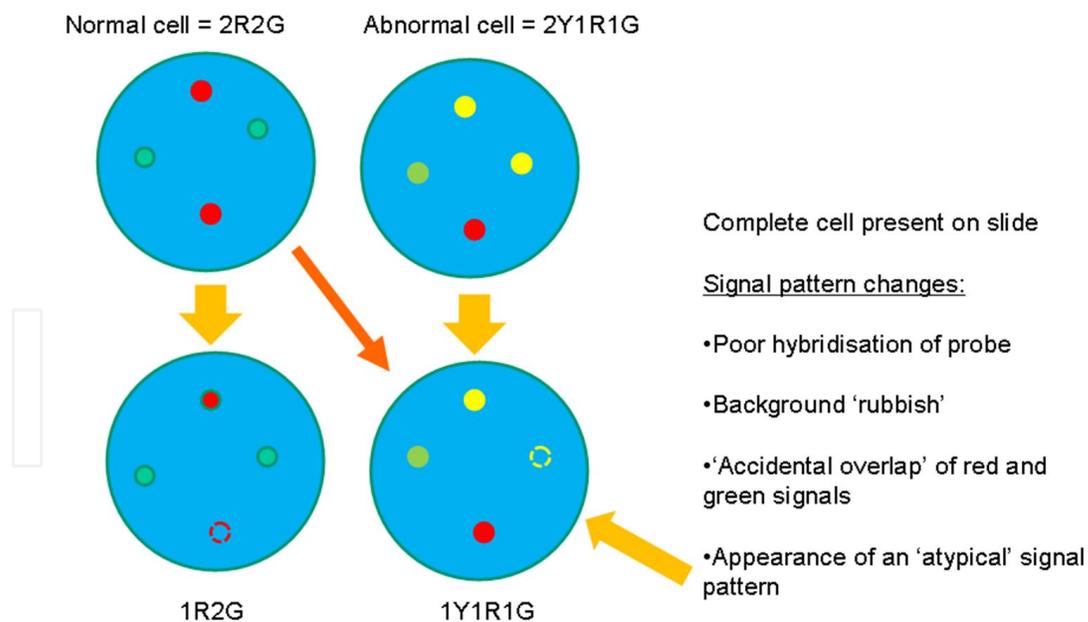


Figure 10. Artefactual signal changes on suspension FISH slides.

Despite such assistance however, care must also be taken during the analysis of paraffin samples, as in many cases it is impossible to completely remove the non-target tissue from the area of interest. It is therefore important to check the H+E slide before beginning the

analysis, as this will give an indication as to whether the sample is made up solely of target tissue, or whether it contains a mixture of target and non-target tissue that must be taken into account when making the final interpretation.

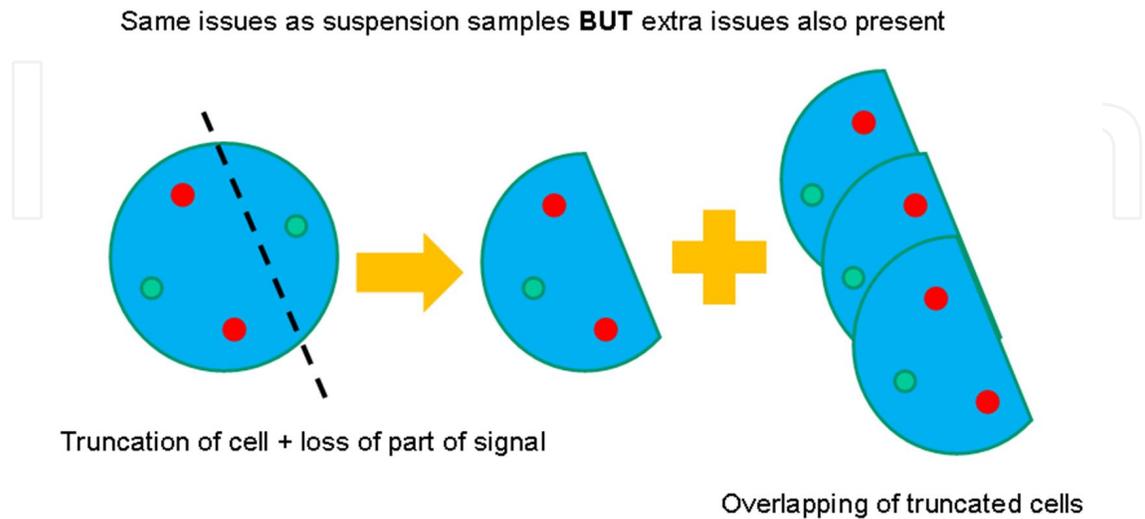


Figure 11. Artefactual considerations for paraffin FISH samples - truncation and overlapping of cells in specimen.

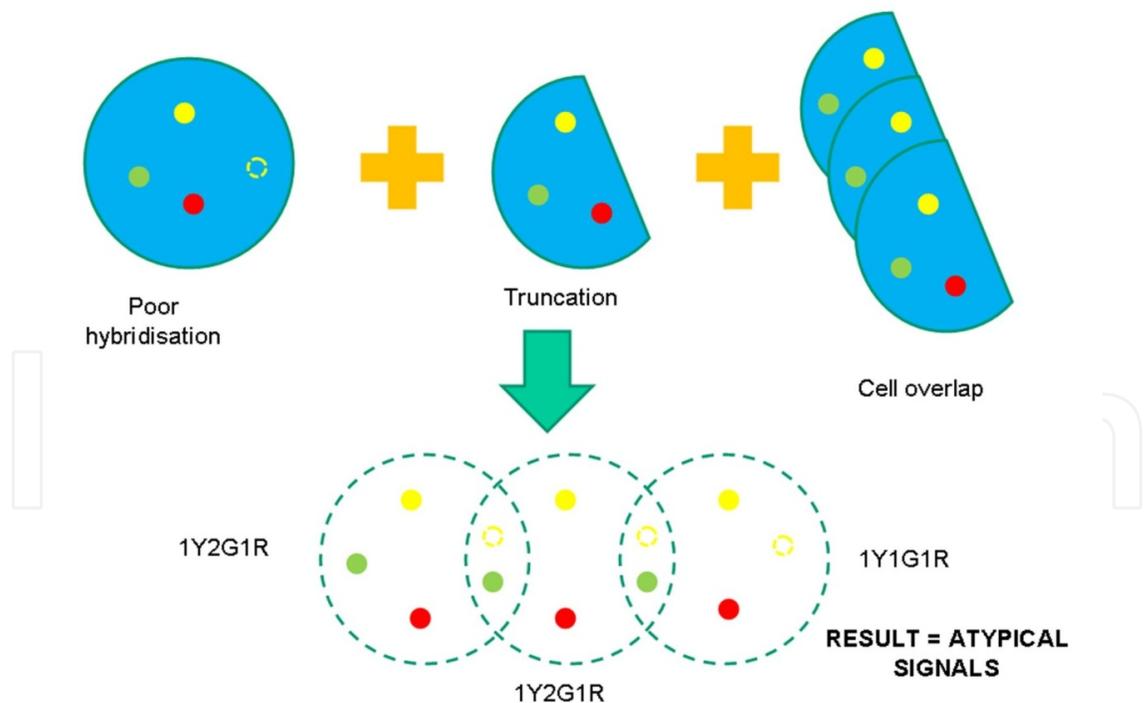


Figure 12. The need for thresholds for paraffin FISH analysis.

Due to both the potential for analysis of the incorrect target cells as outlined, and the artefactual variation that can arise when using the FISH technique [13], it is necessary to establish

robust thresholds to guide the interpretation of results. Signal pattern changes can occur due to poor hybridization of probe, background 'rubbish-autofluorescence' or 'accidental overlap' of red and green signals (Figure 10).

These can lead to the appearance of false or atypical signal patterns; therefore thresholds need to be established to distinguish between false positives and negatives. Paraffin analysis requires higher thresholds than those for suspension cultures, as there is the additional complication of overlap and truncation of cells [1,12], causing artefactual gain or loss of signals (Figures 11 and 12).

Thresholds are of particular importance when dealing with cases that show atypical, non-target (e.g. unexpected loss or increase of copy number instead of a gene rearrangement) or low level abnormalities, or those where mosaicism or multiple clones appear to be present, as it is unclear in most cases as to how they may impact on patient treatment. While paraffin FISH is usually not the most appropriate way to deal with such cases, but when tissue is scarce or has already been processed, it can sometimes be the only option for testing. Numerical scoring of the tissue in such cases will give an indication of the major signal pattern(s) and the level of variation inherent in the tissue, particularly in tumours where there can be concurrent increase in the ploidy level, together with loss or gain of the target loci. This will allow a judgment to be made about whether the variation is likely to be artefactual or not, as false aneuploidies will show almost equivalent levels of loss between target and control loci.

Due to the potential complexities of paraffin analysis, the use of both cytogenetic and pathology external quality control programs such as the College of American Pathologists (CAP) and Australasian Society of Cytogeneticists (ASoC) is recommended, as it allows quality issues to be addressed from both the cytogenetic and pathology perspectives. This provides a balanced perspective on the degree of analytical stringency that is required prior to releasing result.

Appendices

Materials

Reagents

Biotin and Digoxigenin

Bovine serum albumin (BSA) Deionised water

Enzyme reagent (Invitrogen cat #00-8401)

Ethanol (70%, 80% and 100%)

Heat pre-treatment solution pH7.0 (Invitrogen cat #00-8401)

Hybridisation buffer

Non ionic detergent: NP40 (Vysis 30-80482). Store in -20°C.

Phosphate Buffered Saline (PBS)

0.01% pepsin/HCl solution

Purified H₂O

DNA probes

Shandon Rapid-Chrome™ Frozen Section Staining kit

2X SSC/0.1% Tween20

0.4xSSC/0.3%Tween20 solution

2xSSC/0.01% Tween20 solution

Vectashield antifade mounting solution with 1.5µg/ml DAPI (Vector laboratories Cat # H-1200). Store in the dark at 4°C.

Xylol

Equipment

Atlas cooler box

Blotting paper

Centrifuge –Heraeus Biofuge Pico

Coverslips (13mm diameter round, 22x22mm and 24 x 50mm)

Diamond pen or diamond-tipped engraver –Easy Marker Engraver (Taiwan)

Eppendorf tubes

Fix-resistant marker pen

Fluorescence microscope - Zeiss Axio Imager.M1 microscope, Zeiss Axioplan microscope, Olympus BX60 fluorescence microscope

Glass coplin jars

H&E slide

Hotplate

Humidified box

Incubator – Contherm Scientific NZ

Parafilm

Pipettes (2 ul and 100ul)

Pipette tips

Poly-lysine slides (with tissue sections of 2-5µm thickness)

Rubber cement – Weldtite Vulcanising Rubber Solution

Safety goggles

Scalpel

Scissors

Slide drying racks

Thermal cycler – MJC Research PTC- 100 and PTC-200 Peltier Thermal Cyclers

Transmitted light microscope (Zeiss)

Fine tweezers (2 pairs)

Water bath – Grant Instruments (Cambridge)

Recipes

Biotin- and Avidin-conjugated antibodies

Store antibodies as 20 μ l aliquots at 4°C in sterile eppendorf tubes. Do not freeze.

Texas Red Avidin DCS (Biotek/Vector Laboratories Cat #A-2016).

Add 0.5ml of MilliQ water to 1mg lyophilised antibody for a final concentration of 2mg/ml.

Fluorescein Avidin DCS (Cell sorter grade), (Biotek/Vector Laboratories Cat #A-2011).

2mg/ml stock solution aliquotted at 20 μ l and stored in the dark at 4°C. Dilute 1:400 in 4xSSC/1% BSA immediately prior to use.

Biotinylated goat anti-avidin D (Biotek/Vector Laboratories Cat #BA0300).

Add 1ml of MilliQ water to 0.5mg lyophilised antibody for a final concentration of 0.5mg/ml.

Bovine Serum albumin (BSA)

1% BSA in 4xSSC. Dissolve 0.25g of BSA (Sigma A-7030) in 25ml 4xSSC pH 7.0. Store at 4°C for up to 1 month.

FITC– conjugated anti-digoxigenin antibodies

Store antibodies as 50 μ l aliquots at 4°C in sterile eppendorf tubes. Do not freeze.

Anti-digoxigenin-fluorescein, FAB fragments (Boehringer Mannheim Cat #1207741).

Add 1ml of MilliQ water to 200 μ g lyophilised antibody for a final concentration of 0.2mg/ml.

Rabbit fluorescein anti-sheep IgG(H+L) (Biotek/Vector Laboratories Cat #FI-6000).

Add 1ml of MilliQ water to 1.5mg lyophilised antibody for a final concentration of 1.5mg/ml.

Goat fluorescein anti-rabbit IgG(H+L) (Biotek/Vector Laboratories Cat #FI-1000).

Add 1ml of MilliQ water to 1.5mg lyophilised antibody for a final concentration of 1.5mg/ml.

Ethanol 100% Molecular biology grade.

Ethanol 80% Mix ethanol absolute (molecular biology grade) and distilled water in a 4:1 ratio (v/v).

Ethanol 70% Mix ethanol absolute (molecular biology grade) and distilled water in a 7:3 ratio (v/v).

Hydrochloric acid (HCl)

0.2M HCl. Add 2.4ml of 5N HCl to 60mls of MilliQ water.

0.01N HCl. Add 1mL of 5N HCl to 499mLs of distilled water. Store at room temperature for up to 1 year.

Phosphate buffered saline (PBS)

1xPBS. Ca⁺⁺ and Mg⁺⁺ free. Dissolve 8.0g sodium chloride, 0.2g potassium chloride, 2.89g Na₂HPO₄.12H₂O and 0.2g KH₂PO₄ in order in 750ml of MilliQ water. Adjust the volume to 1 litre and autoclave. Store at room temperature.

Pre-treatment reagents for paraffin embedded tissue – Zymed (Invitrogen) Spotlight™HER2 CISH kit (84-0146)

Reagent A. 1 litre of heat pretreatment solution, pH 7.0 (Ready-To-Use).

Reagent B. 5 ml of enzyme pretreatment reagent (Ready-To-Use).

Saline sodium citrate (SSC)

20xSSC (7.0). Dissolve 175.3g sodium chloride and 88.2g trisodium citrate in 800ml MilliQ water. (or use SSC that comes with the Vysis kits; add 4 bottles to make 1L), pH to 7.0 and adjust the final volume to 1 litre. Autoclave and store at room temperature.

4xSSC (pH7.0). Add 200ml of 20xSSC to 700ml MilliQ water. pH to 7.0 and adjust the final volume to 1 litre. Autoclave and store at room temperature.

4XSSC/0.05% Tween20. Add 500µl Tween20 to 1 litre of 4xSSC. Mix well.

2XSSC/0.1% NP40. Add 1mL of NP40 to 1L of 2XSSC (pH7.0)

2xSSC (pH7.0). Add 100ml 20xSSC (pH 7.0) to 800ml MilliQ water. pH to 7.0 and adjust the final volume to 1 litre. Autoclave and store at room temperature.

1xSSC (pH 7.0). Add 50ml of 20xSSC (pH 7.0) to 950ml of milliQ water. Adjust the pH to 7.0, autoclave and store at room temperature.

0.4XSSC/0.3% NP40 (Quickwash buffer). Add 20ml of 20xSSC and 3ml of NP40 to 900ml MilliQ water. Adjust the pH to 7.0 and final volume to 1 litre. Store at room temperature.

Caution

All reagents are potentially hazardous. Appropriate safety procedures must be followed when handling these materials. Avoid contact with skin and mucous membranes, and heating of slides should be performed in a fume hood, as formalin fixed specimens may produce toxic fumes when heated during processing. For more information consult the Hazardous Substances Data Bank (HSDB) - <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>.

Formamide: perform steps involving formamide in hood to avoid inhalation of fumes

Xylene: perform steps involving xylene in hood to avoid inhalation of fumes

Commercial probes and hybridisation buffer solutions: Wear gloves at all times, and when co-denaturing probes use a fume hood, as formamide may be present in probe mixtures and give off toxic fumes.

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