

## REMBRANDT®

*In situ* Hybridisation and Detection


### *DISH & HRP Detection Kit-v11*

| <i>DISH-HRP kit for<br/>the detection of</i> | <b>Biotin Label<br/>product code</b> | <b>Digoxigenin label<br/>product code</b> | <b># Assays</b> |
|----------------------------------------------|--------------------------------------|-------------------------------------------|-----------------|
| <b>HPV screening</b>                         | A100K.0101                           | A100K.9901                                | 10-100          |
| <b>HPV typing</b>                            | A103K.0101                           | A103K.9901                                | 10-100          |

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## Chapter 1 Introduction

### 1.1 Intended use

REMBRANDT® has been designed for the detection of specific DNA or RNA sequences by using the *in situ* Hybridisation (ISH) technique in paraffin embedded tissue sections, cytological specimens and frozen sections.

### 1.2 The ISH principle

ISH enables the detection of specific DNA or RNA sequences in histological and cytological specimens, without losing the often very essential morphological details. The principle of ISH is based on a “reaction” (= hybridisation) between a specifically labelled DNA or RNA sequence (= probe) and a DNA or RNA sequence present in the sample (= target). In case of matching sequences, a hybrid between the probe and target will be formed. Non-perfect matches are washed out by the stringency wash procedure (PanWash). The formed hybrids can easily be visualised by a specific staining procedure, i.e. substrate conversion by enzyme-conjugated antibodies. This conversion, i.e. the combination of AEC and Horseradish Peroxidase (HRP) conjugated anti-DIG or anti-BIO antibodies provided with this kit, will yield a detectable and coloured precipitation. The ISH technique is highly sensitive, specific, fast and easy to perform. Moreover, no radioactivity is involved. The reagents supplied with this kit are tailored to each other and therefore, REMBRANDT® is the ultimate user-friendly tool for performing ISH.

### 1.3 Controls

Use of both positive and negative controls is an essential part of the routine. To ensure that the ISH procedure is performed correctly and that observed positive and/or negative staining are specific, controls should be included in each experiment. This REMBRANDT® kit includes positive and negative control probes serving as a procedure control to be used on sections from the specimen under investigation. The positive control slides contain the desired target DNA and serve as a control for the specific probe. Additional control slides and probes are available from PanPath; please contact your local supplier.

### 1.4 Contents of a REMBRANDT® DISH & HRP Detection Kit

| Item label | description                | Item (cap) colour  | Item contents description                               | Item amount |
|------------|----------------------------|--------------------|---------------------------------------------------------|-------------|
| DIGEST     | PEPSIN POW                 | Transparent vial   | : Pepsin digestion reagent                              | 1 gram      |
| DIGEST     | PEPSIN DIL                 | Transparent vial   | : Pepsin diluent (1M HCl solution)                      | 15 mL       |
| PROBE      | ..... <sup>2</sup>         | Yellow/Purple vial | : Specific <sup>1</sup> BIO or DIG labeled DNA probe(s) | 1 mL        |
| PROBE      | + ..... <sup>2</sup> DISH  | Pink vial          | : DISH positive control oligo probe (BIO or DIG)        | 1 mL        |
| PROBE      | - ..... <sup>2</sup> DISH  | Green vial         | : DISH negative control DNA probe(BIO or DIG)           | 1 mL        |
| PANWASH    | GC% ..... <sup>2</sup> 50% | White vial         | : PanWash (differentiation reagent)                     | 2 x 15 mL   |
| CONJ       | ..... <sup>2</sup> HRP     | Red vial           | : HRP-conjugated anti-DIG or anti-BIO                   | 15 mL       |
| SUBS       | AEC                        | Blue vial          | : AEC substrate                                         | 2 mL        |
| BUFF       | AEC                        | Blue vial          | : AEC buffer                                            | 15 mL       |
| COUNT      | MG                         | Orange vial        | : Methyl Green counterstain                             | 15 mL       |

## ~ Kit contents continued ~

| Item label description |                    | Item (cap) colour | Item contents description             | Item amount |
|------------------------|--------------------|-------------------|---------------------------------------|-------------|
| WASH                   | TBS                |                   | White pouches : TBS buffer salt       | 2 pcs       |
| SUPPORT                | GL SLIDES          |                   | White box A : Coated + glass slides   | 50 pcs      |
| SUPPORT                | COVERSL            |                   | White box B : Coverslips              | 100 pcs     |
| CONTROL                | ..... <sup>2</sup> |                   | White box C : Positive control slides | 2 pcs       |

<sup>1</sup> For specific probe specifications see page 9

<sup>2</sup> Depends on Kit specification

## 1.5 Materials required but not included

- Xylene for dewaxing paraffin sections.
- Fixative for cytological and frozen specimens.
- Distilled or deionised water.
- 100% Ethanol.
- 95% Ethanol.
- 70% Ethanol.
- Water-based mounting medium.
- Pipettes and tips to deliver 10-1000 µL.
- Incubation oven, set at 56-60°C to bake paraffin sections.
- Heating block/slide warmer, set at 37°C.
- Surface thermometer.
- Hotplate, set at 95°C.
- Light microscope for objective 10-100x.

## 1.6 Storage and shelf life

- Store all reagents at 2-8°C upon receipt of the kit.
- Store the dissolved and aliquoted pepsin reagent at -20°C, stable for at least 1 year when kept frozen.
- Store the dissolved TBS buffer at 2-8°C when not in use.
- When used and stored as indicated, the kit is stable until the expiration date printed on the box.

## 1.7 Safety precautions

- Some reagents contain preservatives which can cause irritation when exposed to skin or mucous membranes. The concentrations of these preservatives, however, are very low (< 0.1%). If reagents come into contact with skin or eyes, rinse with large volumes of clean water.
- Never pipette solutions by mouth.
- The control slide in the kit contains pathogenic material fixed in 4% para-formaldehyde making specimens non-infectious; however, we advise taking standard precaution measures for handling infectious organisms.

## 1.8 Performance precautions

- Read all instructions before processing any assay.
- **DO NOT** use reagents beyond their expiry date.
- Allow all components to warm up to room temperature (20-25°C) before use.
- Homogenize probe solution before use.
- Avoid cross contamination of specimens.
- **DO NOT** substitute a reagent with one from another manufacturer.
- When using treated glass slides other than those provided in the kit, specimens may fall off during the procedure.
- **DO NOT** perform the differentiation step on specimens incubated with the positive control oligo probe (pink)!

## 1.9 Preparation of reagents in advance

### Pepsin digestion reagent:

Dissolve the proteolytic reagent in 8 mL of distilled or deionised water (upon receipt of the kit). Aliquot in portions of i.e. 150 µL and store at -20°C.

### Pepsin diluent:

Dilute the 1M HCl solution (transparent) to the application required concentration (paraffin sections 0,1M; cytological and frozen preparations 0,01M) with distilled or deionised water.

### TBS buffer salt:

Dissolve 1 pouch in 1000 mL distilled or deionised water. Dissolve the salt completely and keep the buffer free from contamination.

## 1.10 Preparation of the proteolytic work solution

Prepare proteolytic work solution; 300 to 400 µL per section of 1 cm<sup>2</sup>. Make fresh work solution just before use and discard non-used solution.

### Paraffin sections:

dilute aliquoted proteolytic reagent 100x in 0.1M HCl, e.g. add 100 µL to 5 mL 0.1M HCl and mix.

### Cytological specimens:

dilute aliquoted proteolytic reagent 25,000x in 0.01M HCl, e.g. add 8 µL to 100 mL 0.01M HCl and mix.

### Frozen sections:

Dilute aliquoted proteolytic reagent 50,000x in 0.01M HCl, e.g. add 4 µL to 100 mL 0.01M HCl and mix.

## Chapter 2 REMBRANDT® DISH & HRP Detection Protocol

All incubation steps should be performed in a closed incubation chamber which contains a liquid (water) creating a saturated moisturised environment. During the incubation steps, evaporation of reagents should be prevented. Once the hybridisation procedure has been started the specimen should not be allowed to dry.

### 2.1 Specimen collection and pre-treatment

#### *Paraffin embedded tissue sections*

A standard procedure for tissue fixation and embedding usually involves the use of formalin and paraffin. The optimal tissue block size is 0.5 cm<sup>3</sup>. The formalin should be buffered and fixation times should (preferably) not exceed 12 hours. Excess and/or insufficient fixation may yield suboptimal morphology and target preservation. Embedding in paraffin should not exceed temperatures of 65°C.

Sample preparation: stretch 4 µm paraffin sections on distilled water of 55°C without any additives and collect sections on bio-adhesive (i.e. organosilane) coated glass slides. Bake the slides at 56°C - 60°C in a dry air oven for 2-16 hours. Slides can be used immediately or they can be stored at room temperature for up to 3 months. Prior to ISH, slides need to be dewaxed in fresh xylene for 2 x 10 minutes. Incomplete removal of formalin and/or paraffin may affect the result of the procedure. Remove the xylene by placing the slides in 100% ethanol for 5 minutes. Air dry the slides for approximately 5-10 minutes and start with proteolytic treatment.

#### *Cytological specimens*

Make sure that no multilayer of cells is formed when making a cytological specimen. A multilayer will hamper microscopic examination of the result. The specimen be processed as soon as possible after sampling.

Sample preparation: deposit cells on coated glass slides and air dry for 30 minutes. Fix the cells with a cross-linking fixative (e.g. 4% para-formaldehyde) for 10 minutes at room temperature and rinse with PBS. Dehydrate in graded ethanol series, air dry and start with proteolytic treatment.

#### *Frozen sections*

In general, small pieces of tissue (max. 1 cm<sup>3</sup>) are snap frozen in liquid nitrogen and either stored at -70°C or used immediately. Frozen sections are more fragile than paraffin embedded tissue sections. They should be handled with care and processed as soon as possible.

Sample preparation: collect frozen sections (4 µm) on bio-adhesive (i.e. organosilane) coated glass slides and air dry for 30 minutes. Fix the sections with a cross-linking fixative (e.g. 4% para-formaldehyde) for 10 minutes at room temperature. Dehydrate in graded ethanol series, air dry and start with proteolytic treatment.

### 2.2 Proteolytic treatment

Place both test and control slides on a 37°C heating block or slide warmer and add 300-400 µL of a freshly prepared, pre-warmed proteolytic work solution to each specimen. Incubate at 37°C: paraffin sections for 30 minutes, cytological and frozen specimens for 10 minutes. Tap off proteolytic work solution and dehydrate the slides in graded ethanol series (70%, 95% and 100%). Duration of each soak is 1 minute. Air dry the slides and start with the hybridisation procedure. Do not treat more than 5 slides at the same time, because the temperature of the hot plate may drop dramatically, thus causing incomplete proteolysis.

## 2.3 Hybridisation procedure

### *Denaturation and Hybridisation*

Homogenize probe solutions. Apply 1 drop or 20 µl of probe solution (yellow/purple) to each specimen and the positive control specimen. Apply 1 drop or 20 µl of the negative control probe (green) to each negative procedure control specimen and apply 1 drop or 20 µl of the positive control probe (pink) to each positive procedure control specimen. Cover all specimens with a cover slip (avoid air bubbles!). Place slides on a 95°C hotplate and incubate for 5 minutes (denaturation). Work in a preset order to ensure that all slides have been incubated at 95°C for the exact same time! Do not denature more than 5 slides at the same time, because the temperature of the hot plate may drop dramatically, thus causing incomplete denaturation. Transfer slides into a moist environment and incubate for 16 hours at 37°C (during the hybridisation the minimum temperature should be room temperature and the maximum temperature should be 37°C). Best results are obtained with prolonged incubation time (16 hours).

### *Differentiation (stringency wash) and rinsing*

- Remove coverslips by submerging the slides in TBS buffer. Soak the slides until the coverslips fall off. Rinse the slides in TBS buffer for 10 minutes. Take the slides out, wipe off excess buffer and dry the edges using a lint-free cloth. Please mind **NOT** to perform the differentiation step on specimens incubated with the positive procedure control oligo probe (pink)!
- Apply 5-6 drops of the appropriate PanWash solution (white) to each specimen (except for the positive procedure control) and transfer the slides onto a 37°C heating block or slide warmer. Incubate for 15 minutes at 37°C. Rinse all slides 3x 1 minute in TBS buffer. Wipe off excess reagent and start with the detection and staining procedure.

## 2.4 Detection and staining procedure

Apply 2-3 drops of HRP-conjugate (red) to each specimen and transfer slides onto a 37°C heating block or slide warmer. Incubate for 30 minutes at 37°C. Tap off excess detection reagent and rinse the slides in TBS buffer. Soak 3x 1 minute in TBS buffer, while occasionally shaking the container. Transfer the slides into a container with distilled or deionised water and soak slides for 1 additional minute.

Prepare during the last soak the AEC work solution in a disposable polypropylene tube or suitable glassware by mixing the AEC substrate with the AEC buffer (both blue) according to the volumes given below. Do not make more work solution than necessary as it deteriorates within 3 hours after production. Keep the AEC work solution well protected from the light.

| # specimens | # drops of AEC substrate | volume of AEC buffer |
|-------------|--------------------------|----------------------|
| 1-13        | 4                        | 2 mL                 |
| 14-26       | 8                        | 4 mL                 |
| 27-39       | 12                       | 6 mL                 |
| 40-52       | 16                       | 8 mL                 |

Take the slides out, wipe off excess of water and dry around the edges using a lint-free cloth. Ensure that the specimen on the slide is not disrupted. Apply 2-3 drops of AEC substrate (blue) to each specimen and transfer the slides onto a 37°C heating block or slide warmer. Incubate in the dark for 5-15 minutes at 37°C (examine the colour development every 5 minutes microscopically). Tap off

excess substrate solution and rinse the slides for 3x 1 minute in changes of distilled or deionised water. The slides are now ready to be mounted or counterstained.

## 2.5 Counterstain procedure

When a contrast colour is desired, the slides can be counterstained using Methyl Green (orange). Wipe off excess reagent and apply 2-3 drops of counterstain to each specimen. Incubate for 1 minute (longer incubation is possible and will yield stronger staining). Tap off excess counterstain and rinse the slides briefly in distilled or deionised water. Mount the slides by using an aqueous mounting medium. Interpret the results under the microscope.



## Chapter 3 Limitations of Procedure

### 3.1 Limitations

- The Rembrandt DNA and RNA *in situ* Hybridisation and Detection kits are solely applicable for the detection of corresponding DNA or RNA which may be present in cell preparations (paraffin sections, frozen sections or cytological specimen).
- Appropriate medical decisions are only possible if the medical traceability is ensured. The product is intended for professional use as an aid in the diagnosis corresponding to the DNA or RNA probes as supplied with the kit.
- Either human tissue sections or human cytological preparations may be used. Samples must be fixed in buffered formalin or alcohol. Sections should be cut at 4 µm thickness, glued to the glass slides with a bio-adhesive (e.g. organosilane), dried at room temperature, subsequently dried at 37 °C overnight, complete deparaffinisation in xylene and alcohol series and air dried. Cytological specimen should be prepared as required by the user, fixed with cytological fixation agent, rinsed in distilled water prior to the ISH procedure and air dried.
- Many factors can influence the performance of the ISH procedure. Failure in detection can be due to i.e. improper sampling, handling, the time lapse between tissue removal and fixation, the size of the tissue specimen in the fixation medium, the fixation time, processing fixed tissue, the thickness of the section, the bio-adhesive on the slide, deparaffinisation procedure, incubation times, proteolytic pre-treatment, detection reagents, incubation temperatures and interpretation of results.
- The performance of the ISH procedure is also affected by the sensitivity of the method and the DNA or RNA target load; in case the limit of the sensitivity is reached or when the target DNA or RNA load is too low, a false negative reaction may be the result.
- The clinical interpretation of the results should not be established on the basis of a single test result. A precise diagnosis, in fact, should take into consideration clinical history, symptoms, as well as morphological data. Negative results therefore do not rule out any possibility of a positive specimen.
- The Rembrandt test results are not to be relied on in case the sampling, sampling method, quality, sample preparation, reagents used, controls and procedure followed is not optimal.
- Therapeutic considerations based on the result of this test alone should not be taken. Positive results should be verified by other traditional diagnostic methods such as but not limited to clinical history, symptoms, as well as morphological data.
- The medical profession should be aware of risks and factors influencing the intensity, the absence or presence of probe signals which can not be foreseen when applying this test.
- The user should carefully consider the risk and use of sample material for this test in case the sample material does not contain sufficient or representative test material.
- Laboratory personnel performing the test should be knowledgeable and be able to interpret the test results.

### 3.2 Interpretation of the results

First, check the negative and positive controls that have been incubated with the test slides simultaneously:

- The negative control should be really negative, i.e. not show any localised colour precipitations. If the negative control could be interpreted as being positive, discard the results since no conclusions can be drawn.

- The positive control should show colour precipitations in conformity with the localisation of the target DNA or RNA. The colour should show the proper shade and must be clearly visible in the preferential cell/ tissue type and correspond to the target localisation.

In the test slides, start under low power magnification and focus on localisation and colour to see whether:

- The positivity (colour precipitation) observed is localised in the cell type preferred by the target.
- The colour has the right shade (no endogenous or formalin pigment).

Use high power magnification to see whether:

- The positive staining texture (granular, etc), demarcation and localisation are conform the positive control staining pattern.

#### Product in combination with other devices

The Rembrandt *in situ* Hybridisation and Detection kits are intended for stand-alone usage. The *in vitro* diagnostic is intended to be used in combination with standard formalin fixed, paraffin embedded tissue blocks, standard tissue freezing, tissue sectioning (microtome), standard cytological preparation methods, hot plate(s), stove(s), incubation device(s), water bath(s), temperature and incubation time control(s), and other reagents (not supplied with this reagent) and a microscope. The combination has been tested and validated. Since the standard formalin fixed, paraffin embedded tissue blocks, standard tissue freezing, tissue sectioning (microtome), standard cytological preparation methods, hot plate(s), stove(s), incubation device(s), water bath(s), temperature controls, incubation time control(s) and other not supplied reagents such as but not limited to fixation, embedding and dewaxing reagents and a microscope is not combined with the device as a product, conformity with the essential requirements is not applicable. Assay validation criteria are mentioned in 'Interpretation of the Results' and are also depending on the target load, which may influence the validation criteria.

#### Specifications of the DNA probes:

|             | <u>HPV<sup>1</sup></u> | <u>Positive Control DNA</u> | <u>Negative Control DNA</u> |
|-------------|------------------------|-----------------------------|-----------------------------|
| Specificity | 95%                    | 100%                        | 100%                        |
| Sensitivity | 85%                    | 95%                         | 95%                         |

<sup>1</sup> The specificity of the HPV DNA probes is 100% for the HPV species, but the different specific HPV probe sub-types may show some inter-type cross reactions.

## Chapter 4 References

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## Chapter 5 Probe specifications

### REMBRANDT® Biotin and Digoxigenin<sup>1</sup> labelled DNA probe specifications

| PRODUCT CODE             | LABEL      | DNA PROBE SPECIFICATIONS                                          |                        |                                                                  |                                          |
|--------------------------|------------|-------------------------------------------------------------------|------------------------|------------------------------------------------------------------|------------------------------------------|
|                          |            | Description                                                       | Size                   | Region                                                           | Vector                                   |
| A100P.0100<br>A100P.9900 | BIO<br>DIG | Human papilloma virus HPV screening DNA probe (PROBE xxx panHPV)* | 100-300 bp             | mix of total genomes 7-8 Kb; containing the conserved HPV region | pBR322; 4.3 Kb and pSP; 3.0 Kb           |
| A191P.0100<br>A191P.9900 | BIO<br>DIG | Human papilloma virus type 6/11 DNA probe (PROBE xxx HPV 6/11)*   | 100-300 bp             | total genome 7.8 Kb HPV type 6 and 7.9 Kb HPV type 11            | pSP; 3.0 Kb                              |
| A192P.0100<br>A192P.9900 | BIO<br>DIG | Human papilloma virus type 16/18 DNA probe (PROBE xxx HPV 16/18)* | 100-300 bp             | total genome 7.9 Kb HPV type 16 and 7.9 Kb HPV type 18           | pSP; 3.0 Kb                              |
| A193P.0100<br>A193P.9900 | BIO<br>DIG | Human papilloma virus type 31/33 DNA probe (PROBE xxx HPV 31/33)* | 100-300 bp             | total genome 7.9 Kb HPV type 31 and 7.9 Kb HPV type 33           | pBR322; 4.3 Kb and modified pSP ≈ 4.0 Kb |
| A106P.0100<br>A106P.9900 | BIO<br>DIG | Human papilloma virus type 6 DNA probe (PROBE xxx HPV 6)*         | 100-300 bp             | total genome 7.8 Kb HPV type 6                                   | pSP; 3.0 Kb                              |
| A111P.0100<br>A111P.9900 | BIO<br>DIG | Human papilloma virus type 11 DNA probe (PROBE xxx HPV 11)*       | 100-300 bp             | total genome 7.9 Kb HPV type 11                                  | pSP; 3.0 Kb                              |
| A116P.0100<br>A116P.9900 | BIO<br>DIG | Human papilloma virus type 16 DNA probe (PROBE xxx HPV 16)*       | 100-300 bp             | total genome 7.9 Kb HPV type 16                                  | pSP; 3.0 Kb                              |
| A118P.0100<br>A118P.9900 | BIO<br>DIG | Human papilloma virus type 18 DNA probe (PROBE xxx HPV 18)*       |                        | total genome 7.9 Kb HPV type 18                                  | pSP; 3.0 Kb                              |
| A131P.0100<br>A131P.9900 | BIO<br>DIG | Human papilloma virus type 31 DNA probe (PROBE xxx HPV 31)*       | 100-300 bp             | total genome 7.9 Kb HPV type 31                                  | pBR322; 4.3 Kb                           |
| A133P.0100<br>A133P.9900 | BIO<br>DIG | Human papilloma virus type 33 DNA probe (PROBE xxx HPV 33)*       | 100-300 bp             | total genome 7.9 Kb HPV type 33                                  | modified pSP ≈ 4.0 Kb                    |
| Q001P.0100<br>Q001P.9900 | BIO<br>DIG | Negative control probe for DNA (CONTROL – xxx DISH)*              | 100-300 bp             |                                                                  | pSP; 3.0 Kb                              |
| Q151P.0100<br>Q151P.9900 | BIO<br>DIG | Positive control probe for DNA (CONTROL + xxx DISH)*              | 30-mer oligonucleotide | Mixture of six oligonucleotides complimentary to ALU repeats     |                                          |

\* xxx = label (BIO or DIG)

<sup>1</sup> Digoxigenin (DIG) labeling and detection is protected by international patents of Roche Molecular Biochemicals. This product is supplied under a license of Roche Molecular Biochemicals. This product or the use of this product may be covered by one or more patents of Roche Molecular Biochemicals, including the following: EP patent 0324 474 (granted); U.S. patent 5.354.657 (granted).

|                                                                                  |                                                                                                                                                                                                                                                                                     |
|----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Contents                                                                         | : - clear vial, yellow cap = BIO labelled probe; 1 mL (10-100 assays)<br>- clear vial, purple cap = DIG labelled probe; 1 mL (10-100 assays)<br>- white vial, white cap = matching PanWash; 15 mL<br>R013R.0000 for probes with GC % < 50%<br>R014R.0000 for probes with GC % > 50% |
| Format                                                                           | : ready to use                                                                                                                                                                                                                                                                      |
| Application                                                                      | : colorimetric detection of respective DNA in human specimen by <i>in situ</i> hybridisation (ISH)                                                                                                                                                                                  |
| Purification                                                                     | : by size exclusion chromatography                                                                                                                                                                                                                                                  |
| Detection limit                                                                  | : 3-10 pg by filter hybridisation                                                                                                                                                                                                                                                   |
| Storage                                                                          | : refrigerated (2-8 °C); do not freeze                                                                                                                                                                                                                                              |
| Stability                                                                        | : until expiry date printed on label                                                                                                                                                                                                                                                |
| Precautions                                                                      | : - homogenise solutions before use<br>- avoid contact with eyes and skin; do not swallow                                                                                                                                                                                           |
|  | : ready to use                                                                                                                                                                                                                                                                      |
|  | : harmful: avoid contact with eyes and skin; do not swallow                                                                                                                                                                                                                         |

## Chapter 6      Trouble Shooting Guide

### 6.1 Introduction

This Trouble Shooting Guide is intended to support you in obtaining optimal results with PanPath's REMBRANDT® *In Situ* Hybridisation and Detection kits.

In the next pages we inform you not only about possible causes and remedies for often occurring problems when performing ISH, but we also provide you with some tips given by experts on *In Situ* hybridisation that may be of help to you.

It is of course always possible that you encounter a problem which is not covered by this Trouble Shooting Guide, or that you still have doubts about your results. In such cases, please do not hesitate to contact your local supplier or PanPath directly. Since we consider your problem as our problem, we will do our utmost to find a proper solution.

### 6.2 No section or cells left on the slides

| Possible causes                     | Remedies                                                                                                                                    |
|-------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|
| ■ Sample preparation.               | → Make sure samples are prepared according to protocol, the tissue is fixed in neutral buffered formalin and the slides are air dried well. |
| ■ Tissue section too thin.          | → Optimal thickness of the tissue is 4-6 µm.                                                                                                |
| ■ Wrong (side of) glass slide used. | → Use only organosilane coated glass slides.                                                                                                |
| ■ Pepsin concentration too high.    | → Make sure correct concentration of pepsin is used (depending on type of specimen).                                                        |
| ■ Digestion step too long.          | → Reduce digestion time (15 minutes instead of 30 minutes) or digest at room temperature.                                                   |
| ■ Denaturation.                     | → Make sure temperature is $95 \pm 5^{\circ}\text{C}$ .<br>→ Denature no longer than 5 minutes.                                             |
| ■ Coverslips removed with force.    | → Make sure that slides are soaked for at least 10 minutes in PBS.                                                                          |

### 6.3 Weak or no staining on a suspected positive sample

| Possible causes                              | Remedies                                                                                                                 |
|----------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|
| ■ Tissue fixation.                           | → Only use buffered formalin fixative.                                                                                   |
| ■ Deparaffinization.                         | → Refresh dewax series.                                                                                                  |
| ■ Digestion.                                 | → Make sure correct concentration of pepsin is used.<br>→ Make sure digestion takes place at 37°C.                       |
| ■ Denaturation.                              | → Make sure temperature is 95 ± 5°C.                                                                                     |
| ■ Interfering internal structures of probes. | → In case of RISH procedures, warm up probe solution at 85°C for 5 min. before usage.                                    |
| ■ Hybridisation procedure.                   | → Homogenize probe solution prior to applying probe on the section.                                                      |
| ■ Washing temperature.                       | → Make sure temperature is 37 ± 2°C.<br>→ Depending on GC%, make sure correct PanWash (Differentiation reagent) is used. |
| ■ Detection procedure.                       | → Make sure temperature is 37 ± 2°C.<br>→ Make sure to incubate in the dark.                                             |
| ■ Low amount of target DNA.                  | → Prolong hybridisation.                                                                                                 |
| ■ Colour precipitate rinsed away             | → Make sure that proper rinse and mounting media are used.                                                               |

### 6.4 Negative staining of the positive control

| Possible causes                                                                                                  | Remedies                                                                              |
|------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| ■ Deparaffinization                                                                                              | → Re-fresh dewax series.                                                              |
| ■ Positive control specimen incubated with positive control probe washed with PanWash. (Differentiation reagent) | → Do not use PanWash (Differentiation reagent) on positive control specimen.          |
| ■ Denaturation temperature.                                                                                      | → Make sure temperature is 95 ± 5°C.                                                  |
| ■ Interfering internal structures of probes.                                                                     | → In case of RISH procedures, warm up probe solution at 85°C for 5 min. before usage. |
| ■ Detection procedure.                                                                                           | → Make sure temperature is 37°C ± 2°C.                                                |

## 6.5 Positive staining of the negative control

| Possible causes                                                | Remedies                                                                                                                                  |
|----------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| ■ Drying out of the section.                                   | → Incubate in a moisturised environment.                                                                                                  |
| ■ Washing procedure.                                           | → Make sure temperature is $37 \pm 2^\circ\text{C}$ .<br>→ Depending on GC%, make sure correct PanWash (Differentiation reagent) is used. |
| ■ Contamination with positive control probe or specific probe. | → Make sure that the positive control probe is the latest to be applied to the section.                                                   |

## 6.6 Non-specific background staining

One should always bear in mind that the staining intensity and the level of background (or non-specific) staining may depend on the type of tissue used.

| Possible causes                       | Remedies                                                                                                                                                                         |
|---------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ■ Tissue section too thick.           | → Optimal thickness of the tissue is 4-6 $\mu\text{m}$ .                                                                                                                         |
| ■ Tissue crumbled.                    | → Make sure tissue is stretched completely.                                                                                                                                      |
| ■ Deparaffinization.                  | → Dewax series                                                                                                                                                                   |
| ■ Denaturation temperature too high.  | → Make sure temperature is $95 \pm 5^\circ\text{C}$ .                                                                                                                            |
| ■ Denaturation step too long.         | → Denature no longer than 5 minutes.                                                                                                                                             |
| ■ Drying out of the section.          | → Incubate all procedure steps in a moisturised environment; prevent evaporation                                                                                                 |
| ■ Washing temperature.                | → Make sure temperature is $37 \pm 2^\circ\text{C}$ .                                                                                                                            |
| ■ Substrate incubation step too long. | → Shorten incubation time with 5 minutes.                                                                                                                                        |
| ■ Endogenous peroxidase.              | → Inactivate endogenous peroxidase by incubating tissue sections in 3% $\text{H}_2\text{O}_2/\text{H}_2\text{O}$ for 15 minutes at room temperature prior to the digestion step. |
| ■ Endogenous alkaline phosphatase.    | → Inactivate endogenous alkaline phosphatase by incubating sections in substrate solution to which 4 mg of levamisole is added.                                                  |

## 6.7 Cross Hybridisation

One should always bear in mind that there is a possibility of cross hybridisation between related subtypes and that a patient can be infected with more than one subtype of a virus.



**REFERENCE GUIDE-VS DISH-RP**

- PRETREATMENT OF PARAFFIN SECTIONS**
- Cut 4-6 µm sections and collect on treated glass slides
  - Heat slides
  - Dewax in fresh xylene
  - Soak slides in 100% ethanol and air dry
- PROTEOLYTIC TREATMENT**
- Upon receipt of the kit dissolve pepsin powder in 8 mL distilled/deionized water, aliquot in 150 µL aliquots and freeze at -20°C.
  - Dilute the 1M HCl pepsin diluent (transparent) to the application required concentration (aqueous, cytological or frozen, see 3)
- Dilute thawed proteolytic stock solution in diluted HCl and incubate each specimen with 300-400 µL:
    - HEPATITIS:** 100x in 0.1M HCl
    - HEPATOMA:** 100 µL to 5 mL 0.1M HCl
    - SARCOID:** 25,000x in 0.01M HCl
    - ESL:** 6 µL to 100 mL 0.01M HCl
    - ITZGAZI:** 50,000x in 0.01M HCl
 add 4 µL to 100 mL 0.01M HCl
  - Discard excess proteolytic work solution
  - Dehydrate slides in graded ethanol and air dry

**INCUBATION TIME**

- 2-16 hours at 56-60°C
- 2 x 10 min.
- 5 min.

**VIAL LABEL**

**ANLEITUNG-VS DISH-RP**

- HERSTELLUNG VON PARAFFINSCHNITTEN**
- 4-6 µm Schnitte anfertigen und auf vorbereitete Objektträger ziehen
  - Schnitte inubieren
  - in Xylol einparaffinieren
  - Schnitte in 100% Ethanol entwässern und lufttrocknen

**INKUBATIONSEITEN**

- 2-16 Stunden bei 56-60°C
- 2 x 10 Min.
- 5 Min.

**DIAGNOSTIK**

**HEPATITIS**

**HEPATOMA**

**SARCOID**

**ESL**

**ITZGAZI**

**HEPATITIS**

**HEPATOMA**

**SARCOID**

**ESL**

**ITZGAZI**

**HYBRIDIZATIONPROCEDURE**

- Apply 1 drop or 20 µL of probe solution per specimen; cover with coverslip
- Denature
- Hybridize
- Remove coverslips by soaking slides in TBS buffer
- Apply 5-6 drops of Parafix (white) to each specimen except the positive control
- Wash all slides in TBS buffer

**PROBE**

- 5 min. at 95°C heating block
- 16 hours at 37°C incubator
- 10 min.
- 15 min. on a 37°C heating block
- 3 x 1 min.

**WASHER**

**TBS**

**WASHER**

**TBS**

**CONJUGATE**

**HRP**

**WASHER**

**TBS**

**WASHER**

**TBS**

**DETECTION AND STAINING PROCEDURE**

- Apply 2-3 drops of the conjugate (red) to each specimen
  - Soak slides in TBS buffer
  - Soak slides in distilled/deionized water
  - Prepare AEC (blue) work solution according the following table
- | Number of specimens | Number of drops of AEC substrate | Vol. of AEC buffer |
|---------------------|----------------------------------|--------------------|
| 1-13                | 4                                | 2 mL               |
| 14-26               | 8                                | 4 mL               |
| 27-39               | 12                               | 6 mL               |
| 40-52               | 16                               | 8 mL               |
- Apply 2-3 drops of AEC work solution to each specimen and incubate in dark

**30 min. on a 37°C heating block**

- 3 x 1 min.
- 1 min.

**DIAGNOSTIK**

**HEPATITIS**

**DIAGNOSTIK**

**HEPATOMA**

**DIAGNOSTIK**

**SARCOID**

**DIAGNOSTIK**

**ESL**

**DIAGNOSTIK**

**ITZGAZI**

**HYBRIDISIERUNGSPROZEDUR**

- Tropfen oder 20 µL der Sonde auf jedes Präparat geben und mit einem Deckglas abdecken
- Denaturieren
- Hybridisieren
- Entfernen der Deckglas durch Einhauchen in TBS Puffer
- 5-6 Tropfen Parafix (Weiß) zu jedem Präparat geben (mit Ausnahme der Positivkontrolle)
- Präparate in TBS Puffer spülen

**PROBE**

- 5 min. bei 95°C Heizplatte
- 16 Stunden bei 37°C Ofen
- 10 Min.
- 15 Min. bei 37°C Heizplatte
- 3 x 1 Min.

**WASHER**

**TBS**

**WASHER**

**TBS**

**WASHER**

**TBS**

**DETEKTION UND FÄRBEPROZEDUR**

- 2-3 Tropfen Konjugat (Rot) auf jedes Präparat geben
  - Präparate in TBS Puffer spülen
  - Präparate mit destilliertem/deionisiertem Wasser spülen
  - AEC (blau) Gebrauchslösung nach nachrichtigem Schema vorbereiten
- | Präparate/Aliquot | Tropfen der AEC Substratlösung | Vol. AEC-Diluent |
|-------------------|--------------------------------|------------------|
| 1-13              | 4                              | 2 mL             |
| 14-26             | 8                              | 4 mL             |
| 27-39             | 12                             | 6 mL             |
| 40-52             | 16                             | 8 mL             |
- 2-3 Tropfen der AEC Gebrauchslösung zu jedem Präparat geben
  - in dunklen inubieren
  - in destilliertem/deionisiertem Wasser spülen
  - Ödnatol: 2-3 Tropfen der Gegenfärbelösung (Orange) auf jedes Präparat geben
  - in destilliertem/deionisiertem Wasser spülen
  - Präparate für die mikroskopische Beurteilung abdecken

**30 Min. bei 37°C Heizplatte**

- 3 x 1 Min.
- 1 Min.

**DIAGNOSTIK**

**HEPATITIS**

**DIAGNOSTIK**

**HEPATOMA**

**DIAGNOSTIK**

**SARCOID**

**DIAGNOSTIK**

**ESL**

**DIAGNOSTIK**

**ITZGAZI**



**GUIDE REFERENCE-V5 DISH-HRP**

**VIAL-LABEL**

**GUIA DE REFERENCIA-V5 DISH-HRP**

- PREPARATION DES SECTIONS PARAFFINES**
1. Préparer des sections de 4-6 µm et colorer les lames traitées
  2. Colorifier les lames
  3. Déparaffiner dans du xylène frais
  4. Immerger dans de l'éthanol 100% (ou éthanol absolu) et laissez sécher à l'air

**TRATAMIENTO PROTICOLOGICO**

1. Dissolvez la poudre de pepsine dans 8 ml de'eau distillée ou déminéralisée, divisez la solution en aliquotes de 150 µl et congeler les (-20°C).
2. Diluez la solution HCl 1M (solution de pepsine en tant que diluant transparent) selon l'usage préféré (paraffine, cytologique ou congelé)

3. Diluez une aliquote de la solution du stock (proteolytique avec la solution diluée de HCl et incubez chaque échantillon dans 300-400 µl de la manière suivante:

|                                                 |                             |
|-------------------------------------------------|-----------------------------|
| dilution paraffine: 100x dans 0.1M HCl;         | 30 min, 37°C bloc chauffant |
| ajouter 100 µl à 5 ml de HCl 0.01M              | 3 x 1 min.                  |
| dilution cytoloculaire: 25.000x dans 0.01M HCl; | 10 min, 37°C bloc chauffant |
| ajouter 8 µl à 100 µl de HCl 0.01M              | 10 min, 37°C bloc chauffant |
| dilution congelée: 50.000x dans 0.01M HCl;      | 10 min, 37°C bloc chauffant |
| ajouter 4 µl à 100 µl de HCl 0.01M              | 3 x 1 min.                  |

**PROTOCOLE D'HYBRIDATION**

1. Ajouter une goutte ou 20 µl d'une solution de sonde par échantillon et ouvrir avec une lamelle
2. Déshydrater
3. Hydrater
4. Diffuser verticalement le tampon TBS sur les lamelles
5. Appliquez 5-6 gouttes de PanPath (bleu) à chaque échantillon à l'exception du contrôle positif
6. Rincez toutes les lames avec le tampon TBS

**PROTOCOLE DE DETECTION DE COLORATION**

1. Distribuez 2-3 gouttes de conjugué (orange) par spécimen
2. Rincez les lames dans le tampon TBS
3. Immergez les lames dans de l'eau distillée ou déminéralisée
4. Préparez la solution d'usage AEC (bleu) à partir du biseau ci-dessous:

|                 |                             |                             |
|-----------------|-----------------------------|-----------------------------|
| No. Échantillon | No. gouttes de AEC substrat | Vol. de solution tampon AEC |
| 1-13            | 2                           | 2 ml                        |
| 14-26           | 8                           | 4 ml                        |
| 27-39           | 12                          | 6 ml                        |
| 40-52           | 16                          | 8 ml                        |

5. Ajoutez 2-3 gouttes de la solution d'usage AEC par spécimen et rincez dans l'oscurité
6. Rincez les lames avec de l'eau distillée ou déminéralisée
7. **Optionnel:** ajoutez 2-3 gouttes de la solution colorante (orange)
8. Rincez les lames avec de l'eau distillée ou déminéralisée
9. Montrez les sections et examinez les lames au microscope

- TEMPERATURE D'INCUBATION**
- 2-16 hrs à 56-60°C  
2 x 10 min.  
5 min.

|               |               |            |
|---------------|---------------|------------|
| <b>DIGEST</b> | <b>PERSON</b> | <b>POU</b> |
| <b>DIGEST</b> | <b>PERSON</b> | <b>DUI</b> |

**TRATAMIENTO PROTICOLOGICO**

1. A la recepción del kit disolver la pepsina en polvo en 8 ml de agua destilada/deionizada, hacer alícuotas de 150 µl y congelar a -20°C.
2. Diluir el diluyente de la pepsina (vial transparente) CH 1M a la concentración requerida para la aplicación (parafina, citología o congelación, ver 3)

3. Diluir la solución proteolítica stock descongelada en CH diluido e incubar cada muestra con 300-400 µl:

|                                  |                                    |
|----------------------------------|------------------------------------|
| Parafina: 100x en CH 0.1M;       | 30 minutos en un tembloque a 37°C. |
| añadir 100 µl a 5 ml de CH 0.1M  | 3 x 1 minuto                       |
| Citología: 25.000x en CH 0.01M;  | 10 minutos en un tembloque a 37°C  |
| añadir 8 µl a 100 µl de CH 0.01M | 10 minutos en un tembloque a 37°C  |
| Congelada: 50.000x en CH 0.01M;  | 10 minutos en un tembloque a 37°C  |
| añadir 4 µl a 100 µl de CH 0.01M | 3 x 1 minuto                       |

**PROTOCOLE D'HYBRIDATION**

1. Ajouter 1 goutte ou 20 µl de la solution de la sonde par muestra.
2. Colorer avec un cube.
3. Déshydrater
4. Hydrater
5. Rincer les cubes en immergeant les portes en tampon TBS
6. Appliquez 5-6 gouttes de Pan Wash (vial bleu) à chaque muestra excepto al control positivo
7. Laver todos los portes en tampon TBS

**PROTOCOLE DE DETECCION Y TINCION**

1. Añadir 2-3 gotas de conjugado (vial rojo) a cada muestra
2. Enjuagar las portas en tampon TBS
3. Sumergir las portas en agua desionizada
4. Preparar el AEC a la solución de trabajo de acuerdo con la siguiente tabla:

|          |                        |                       |
|----------|------------------------|-----------------------|
| Muestras | Gotas de sustituto AEC | Volumen de tampon AEC |
| 1-13     | 4                      | 2 ml                  |
| 14-26    | 8                      | 4 ml                  |
| 27-39    | 12                     | 6 ml                  |
| 40-52    | 16                     | 8 ml                  |

5. Añadir 2-3 gotas de AEC a la solución de trabajo a cada muestra e incubar en oscuridad
6. Eliminar el exceso de solución de sustituto y lavar las portas en agua destilada o desionizada
7. **Optionalmente:** añadir 2-3 gotas de solución de contraste (vial naranja) a cada muestra
8. Lavar las portas en agua desionizada
9. Montar las portas para su evaluación por microscopia

**TIEMPO DE INCUBACION**

2-16 horas a 56-60°C  
2 x 10 minutos  
5 minutos

|               |            |
|---------------|------------|
| <b>CONTIN</b> | <b>WIG</b> |
|---------------|------------|

**HANDLEIDING-VS DISH-HRP**

**SNIJDEN EN PAKKEN VAN/PARAFFINE COUPES**

1. Snij 4,5 µm coupes en pak ze op voorbehandelde olieplaatjes
2. Verpak de glasjes.
3. Deparemeer in versje xylol
4. Spoel in 100% ethanol en laat de gasfases luchtrogen

**PROTOCOLYSE VAN/OEBEHANDLING**

1. Los het peptise poeder 8 mL gedestilleerd/gedeïoniseerd water op, verdeel in 150 µL porties en bewaar bij -20 °C.
2. Verdun het 1M HCl peptise oplosmiddel (transparant) bij de concentratie die voor de applicatie nodig is (paraffine, cytologie of vers coupes, zie 3)
3. Verdun de protolytische stock oplossing in het verdunde HCl en incubeer elk preparaat met 300-400 µL.:  
**paraffine:** 100x in 0,1M HCl;  
 voeg 100 µL aan 5 mL 0,1M HCl toe  
**cytologie:** 25.000x in 0,01M HCl;  
 voeg 8 µL aan 100 mL 0,01M HCl toe  
**vers:** 50.000x in 0,01M HCl;  
 voeg 4 µL aan 100 mL 0,01M HCl toe  
 Overmaat van de protolytische werkoplossing weggoeden (alijd vers beelden)
4. Overmaat van de protolytische werkoplossing weggoeden (alijd vers beelden)
5. Onwater de preparaten in oplopende alcoholreeks en luchtrogen

**HERBIDS/TIE PROCEDURE**

1. Incubeer elk preparaat met probe reagens: 1 druppel of 20 µL in dek of met deklglasje
2. Deteriueer
3. Hydriseer
4. Verwijder deglasje door preparaten in TBS buffer te dompelen
5. Incubeer elk preparaat met Pen/Albi (wit), 5-8 druppels
6. Met uitbreiding van de positieve controle
7. Spoel alle preparaten in TBS buffer

**DETECTIE EN VERKLEURINGSPROCEDURE**

1. Incubeer elk preparaat met conjugaat (rood): 2-3 druppels
  2. Incubeer elk preparaat met TBS buffer
  3. Spoel de preparaten met gedestilleerd/gedeïoniseerd water
  4. Bereid AEC (blauw) werkoplossing volgens onderstaande tabel
- | Aantal druppels AEC substraat | Volume AEC buffer |      |
|-------------------------------|-------------------|------|
| 1-13                          | 4                 | 2 mL |
| 14-26                         | 8                 | 4 mL |
| 27-39                         | 12                | 6 mL |
| 40-52                         | 16                | 8 mL |

5. Incubeer elk preparaat met AEC werkoplossing: 2-3 druppels en incubeer in donker
6. Spoel de preparaten met gedestilleerd/gedeïoniseerd water
7. **Donker:** Incubeer elk preparaat met legenkleur (oranje): 2-3 druppels
8. Spoel de preparaten met gedestilleerd/gedeïoniseerd water
9. Dek de coupes af

**INCUBATIE TIDEN**

- 2-16 uur bij 36-50°C
- 2 x 10 min.
- 5 min.

**DIGESTIE PEP SIN ETOH**

- 30 min. bij 37°C hele plaat
- 10 min. bij 37°C hele plaat
- 10 min. bij 37°C hele plaat
- 3 x 1 min.

**RODE**

- 5 min. bij 95°C hele plaat
- 16 uur bij 37°C stof
- 10 min.
- 15 min. bij 37°C hele plaat
- 3 x 1 min.

**WASH TBS**

- 3 x 1 min.
- 3 x 1 min.
- 3 x 1 min.

**CONJUG**

- 3 x 1 min.
- 3 x 1 min.
- 3 x 1 min.

**VAL-LABEL**

**METHODICA D'USO-VS DISH-HRP**

**PRETRATTAMENTO DELLE SEZIONI IN PARAFFINA**

1. Tagliare sezioni di 4-6 µm e depositarle su vetrino trattato
2. Scaldare i vetrini
3. Togliere la paraffina usando xiblo fresco
4. Sciogliere i vetrini in etanolo 100% ed asciugare all'aria

**TRATTAMENTO PROTOLITICO**

1. Seguire le istruzioni del kit, sciogliere la peptisa in polvere in 8 mL di acqua distillata/deionizzata, quindi preparare aliquoti di 150 µL e congelare a -20°C.
2. Diluire la peptisa 1M HCl (trasparente) diluente sino a raggiungere la concentrazione richiesta dal applicatore (Paraffinati, Citologici o Congiati, vedi punto 3)
3. Diluire la soluzione protolitica scoggerata in HCl diluito secondo lo schema seguente (Incubare ogni sezione con 300-400 µL):  
**Paraffinati:** 100x in 0,1M HCl;  
 aggiungere 100 µL a 5 mL 0,1M HCl  
**Citologici:** 25.000x in 0,01M HCl;  
 aggiungere 8 µL a 100 mL 0,01M HCl  
**Congiati:** 50.000x in 0,01M HCl;  
 aggiungere 4 µL a 100 mL 0,01M HCl
4. Gettare i focolacci della soluzione protolitica
5. Disidratate le sezioni in etanolo puro ed asciugare all'aria

**PROCEDIMENTO DI IRRADIAZIONE**

1. Aggiungere 1 goccia o 20 µL di soluzione "probe" su ogni sezione.
2. Coprire con coprivetro.
3. Deteriueare
4. Irradiare il coprivetro scaldando il vetrino in tempore TBS
5. Aggiungere 5-8 goccie di Pen/Albi (bianco) su ogni sezione tirare su controllo positivo
6. Lavare tutti i vetrini in tempore TBS

**PROCEDIMENTO DI DEFEZIONE E COLORAZIONE**

1. Aggiungere ad ogni sezione 2-3 goccie di conjugato (rosso)
  2. Sciogliere i vetrini in tempore TBS
  3. Sciogliere i vetrini in acqua distillata/deionizzata
  4. Preparare la soluzione di lavoro dell'AEC (blu) come segue:  
 No. di sezioni No. di goccie di subistrato AEC Volume in lamina AEC
- |       |    |      |
|-------|----|------|
| 1-13  | 4  | 2 mL |
| 14-26 | 8  | 4 mL |
| 27-39 | 12 | 6 mL |
| 40-52 | 16 | 8 mL |

5. Aggiungere ad ogni sezione 2-3 goccie di soluzione di lavoro AEC e incubare al buio
6. Togliere l'eccesso di soluzione substrato e lavare i vetrini in acqua distillata/deionizzata
7. **Donazione:** aggiungere ad ogni sezione 2-3 goccie di "counterstain" (arancione)
8. Lavare le sezioni in acqua distillata/deionizzata
9. Preparare le sezioni per l'osservazione al microscopio

**TEMPI DI INCUBAZIONE**

- 2-16 ore a 36-50°C
- 2 x 10 min.
- 5 min.

**30 min. su blocco riscaldate a 37°C**

- 10 min. su blocco riscaldate a 37°C
- 10 min. su blocco riscaldate a 37°C
- 3 x 1 min.

**5 min. su blocco riscaldate a 95°C**

- 16 ore a 37°C in incubance
- 10 min.
- 15 min. su blocco riscaldate a 37°C
- 3 x 1 min.

**30 min. su blocco riscaldate a 37°C**

- 3 x 1 min.
- 1 min.
- 30 min. su blocco riscaldate a 37°C

**3 x 1 min.**

- 1 min.
- 3 x 1 min.
- 5-15 min. su blocco riscaldate a 37°C



**ΟΛΗΤΟΣ ΑΝΑΦΟΡΑΣ v-6-DISH-HRP**

**ΧΡΟΝΟΣ ΕΠΙΜΟΡΦΗΣ**

- ΠΡΟΤΟΜΑΚΑΙΑ ΤΟΜΟΝ ΠΑΡΑΦΟΡΗΣ**
1. Κόψτε τμήμα τρυφών 4-6 μm και επηρεώστε σε επηρεωτικές υδατικές ομοιομορφικές υλικές.
  2. Αποξηλώστε σε βέλβου.
  3. Αποξηλώστε σε βέλβου.
  4. Επιστημώστε τις τρυφές σε 100% αλκοόλη και επηρεώστε

**ΠΡΟΤΕΙΝΟΜΕΝΗ ΠΡΟΚΑΤΑΡΑΧΗ**

1. Αποξηλώστε την προκαταρτά του κτ. βέλβου την προκλήσε σε 8 ml ομοιομορφικό / ομοιομορφικό υγρό. Προκαταρτά σε βέλβου και 150 μl και κενώστε τρυφές -20°C
2. Αποξηλώστε το βέλβου σε βέλβου της τρυφής σε 1M HCl, ομοιομορφικό με την επιθυμητή ομοιομορφική για τις επηρεωτικές (σε τρυφών, κενώστε/ομοιομορφικό ή κενώστε/ομοιομορφικό βέλβου).
3. Αποξηλώστε με επηρεωτικό βέλβου 3) ομοιομορφικό βέλβου του ημερησίου ομοιομορφικού σε επηρεωτικό HCl και επηρεώστε κέρβου με 300-400 μl .

- βέλβου σε τρυφών 100 σε 0,1 M HCl  
 Προκαταρτά 100 μl σε 5 ml 0,1 M HCl  
 Κενώστε/ομοιομορφικό βέλβου 25.000x σε 0,01 M HCl  
 Προκαταρτά 8 μl σε 100 ml 0,01 M HCl  
 Κενώστε/ομοιομορφικό βέλβου 50.000x σε 0,01 M HCl

1. Προκαταρτά 4 μl σε 100 ml 0,01 M HCl
2. Αποξηλώστε με επηρεωτικό βέλβου ημερησίου ομοιομορφικού
3. Αποξηλώστε με επηρεωτικό βέλβου και επηρεώστε τις τρυφές

**ΔΙΑΔΙΚΑΣΙΑ ΥΠΕΡΔΙΟΡΘΩΣΗΣ**

1. Προκαταρτά 1 ομοιομορφικό 20 μl από το βέλβου του ομοιομορφικού από βέλβου.
2. Αποξηλώστε
3. Υπερδιόρθωση
4. βέλβου 99°C
5. Προκαταρτά 5-6 ομοιομορφικές βέλβου (βέλβου) σε κέρβου βέλβου, κέρβου του βέλβου ημερησίου
6. Εκμηλώστε βέλβου του ημερησίου σε βέλβου TBS

**ΔΙΑΔΙΚΑΣΙΑ ΑΝΙΧΝΕΥΣΗΣ ΚΑΙ ΠΡΟΚΑΤΑΡΑΧΗΣ**

1. Προκαταρτά 2,3 ομοιομορφικές ομοιομορφικού ομοιομορφικού
  2. Επιστημώστε το ημερησίου σε βέλβου TBS
  3. Επιστημώστε το ημερησίου σε ομοιομορφικό υγρό
  4. Προκαταρτά το βέλβου ομοιομορφικού AEC (υγρό) ομοιομορφικό με τον ομοιομορφικό ημερησίου # βέλβου. # ομοιομορφικού ομοιομορφικού AEC
- |       |    |      |
|-------|----|------|
| 1-13  | 4  | 2 ml |
| 14-26 | 8  | 4 ml |
| 27-38 | 12 | 6 ml |
| 39-52 | 18 | 8 ml |
5. Προκαταρτά 2,3 ομοιομορφικές βέλβου ομοιομορφικού AEC σε κέρβου βέλβου του ημερησίου σε βέλβου
  6. Αποξηλώστε το ημερησίου βέλβου ημερησίου και βέλβου
  7. Διαδοχικά: Προκαταρτά 2,3 ομοιομορφικές βέλβου ομοιομορφικού (προκαταρτά) σε κέρβου βέλβου
  8. Εκμηλώστε το ημερησίου σε ομοιομορφικό υγρό
  9. Εκμηλώστε τις τρυφές για ημερησίου βέλβου

|        |             |
|--------|-------------|
| DETECT | PERSON ONLY |
| ROSETT | PERSON ONLY |

|        |             |
|--------|-------------|
| ROSETT | PERSON ONLY |
|--------|-------------|

|      |                  |
|------|------------------|
| WASH | TBS              |
| WASH | 5% BSA / 40% TBS |
| WASH | TBS              |

|      |     |
|------|-----|
| CONU | HRP |
| WASH | TBS |
| WASH | TBS |
| WASH | TBS |

|       |    |
|-------|----|
| COUNT | MG |
|-------|----|

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