# REMBRANDT®

*In situ* Hybridization and Detection

## *DISH detection assays*-*v5.2023*

| DISH kits | | | |
|---|---|---|---|
| **Detection of** | **Label/Detection** | **Product number** | **# assays** |
| HPV screening | BIO/HRP | A100K.0101 | 10-100 T |
| HPV screening | DIG/HRP | A100K.9901 | 10-100 T |
| HPV screening | BIO/AP | A100K.0105 | 10-100 T |
| HPV screening | DIG/AP | A100K.9905 | 10-100 T |
| HPV typing | BIO/HRP | A103K.0101 | 10-100 T |
| HPV typing | DIG/HRP | A103K.9901 | 10-100 T |
| HPV typing | BIO/AP | A103K.0105 | 10-100 T |
| HPV typing | DIG/AP | A103K.9905 | 10-100 T |

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IVD



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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* Hybridization (ISH) technique in cytological specimen or FFPE tissue sections. In extensive tests and field application, the REMBRANDT® DISH kits were proven to be very robust, and ISH results were highly reproducible. For the specific intended use of a REMBRANDT® DISH detection assay, please see the product specific datasheet.

## 1.2 The DISH principle

DISH enables the detection of specific DNA sequences in histological and cytological specimens, without losing the often very essential morphological details. The principle of DISH is based on a "reaction" (= hybridization) between a specifically conjugated oligonucleotide sequence (= probe) and an DNA sequence present in the sample (= target). In case of matching sequences, a hybrid between the probe and target will be formed. The formed hybrids can easily be visualized after detection with corresponding antibodies by brightfield microscopy. The REMBRANDT® DISH probes are conjugated to biotin or digoxigenin; the conjugate used in the kits depend on specific probe(s) in the kit (Arsham et al., 2017).

## 1.3 Controls

Use of 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 by the end-user.

## 1.4 Contents of a REMBRANDT® DISH detection assay

| Item label description | | Item (cap) colour | Items contents description | Item amount |
|---|---|---|---|---|
| DIGEST | PEPSIN POW | Transparant vial | Pepsin digestion reagents | 1 gram |
| DIGEST | PEPSIN DIL | Transparent vial | Pepsin diluent | 15 ml |
| PROBE | …..[2] | Yellow/purple cap | Specific BIO or DIG labeled probe mix | 1 ml |
| PROBE | + \| ..[2] DISH | Pink cap | DISH positive control oligo probe (BIO or DIG) | 1 ml |
| PROBE | - \| ..[2] DISH | Green cap | DISH negative control oligo probe (BIO or DIG) | 1 ml |
| CONJ | …..[2] HRP or AP.. | Red cap | HRP or AP conjugated anti BIO or anti DIG | 15 ml |
| PANWASH | GC% …50% | White cap | PanWash 50% GC concent | 2x 15 ml |
| SUBS | AEC [2] | Blue cap | AEC substrate | 2 ml |
| BUFF | AEC or NBT/BCIP [2] | Blue cap | AEC buffer or NBT/BCIP substrate/buffer | 15 ml |
| COUNT | MG or FR [2] | Orange cap | Methyl green or Fast red counterstain | 15 ml |
| WASH | TBS | White pouches | TBS buffer salt | 2 pcs |
| SUPPORT | GL SLIDES | White box A | Coated glass slides | 50 pcs |


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| | | | | |
|---|---|---|---|---|
| SUPPORT | COVERSLIPS | White box B | Coverslips | 100 pcs |
| CONTROL | QC SLIDES | Transparant box | Positive control slides | 2 pcs |

1 For specific probe specification see product specific datasheet

2 Depends on kit specification

## 1.5 Probe specifications

REMBRANDT® Biotin and Digoxigenin[1] 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 oligonucleotid | Mixture of six oligonucleotides complimentary to ALU repeats | |

* xxx = label (BIO or DIG)

**Format**: ready to use

**Application**: colorimetric detection of respective DNA in human specimens by *in situ* hybridization (ISH)

**Detection limit**: 10-30 pg by filter hybridization

**Storage**: refrigerated (2-8 °C); do not freeze

**Stability**: until expiry date on label

**Precautions:**

- ear gloves and treat glassware overnight at 200°C before use
- Homogenize solutions before use

## 1.5 Materials required but not included

- Xylene for dewaxing paraffin sections
- Fixative for cytological specimens
- Distilled or deionised water
- Ethanol series
  - 100% Ethanol
  - 96% Ethanol
  - 70% Ethanol
- Water based mounting medium
- Pipettes and tips to deliver 10-1000 µL
- Immersion oil for brightfield microscopy
- Timer(s)
- Microwave (optional)
- Mircoscope and accessories
- Oven(s), heating block(s) and water baths
- Incubation chamber
- Mixing device i.e. vortex
- Magnetic stirrer
- (Surface) thermometer; calibrated
- pH meter and calibrators
- Coverslips
- Slides

## 1.6 Storage and shelf life

– Store kit and its contents at 2-8°C.
– Store the dissolved and aliquoted reagents 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 expiry date printed on the box.

## 1.7 Safety precautions

– If reagents come into contact with skin or eyes, rinse with large volumes of clean water.
– Never pipette solutions by mouth.
– All hazardous materials should be disposed of according to national guidelines.
– A material safety data sheet is available on request

## 1.8 Performance precautions

– Read all instructions before processing any assay.
– Store all reagents as recommended.
– ***DO NOT*** use reagents beyond their expiry date.
– Allow all components to warm up to room temperature (20-25°C) before use.



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- Check all ready to use solutions for precipitates and other contaminations before use; if contaminations are present, the solution should be disposed of, and a new solution should be prepared.
- PanWash 4 concentrated solution may form crystals when stored at low temperatures. If heating does not dissolve the crystals, the solution should be disposed of and a new solution should be prepared.
- Homogenize all solutions before use.
- Homogenize probe solution and spin briefly before use.
- ***DO NOT*** substitute a reagent with one from another manufacturer.
- Use treated glass slides, preferably REMBRANDT® Adhesive coated glass slides (Z000S.0002, PanPath B.V.).
- ***DO NOT*** re-use prepared, ready to use digestion reagent; dispose of residuals.
- With every new procedure, check the temperatures of pre-treatment solutions, denaturation device used, incubation device used and PanWash solution by using a **calibrated** thermometer.
- Do not incubate more than 5 specimens at the same time in pre-set temperature baths/devices. Placing more than 5 specimens in such a device will cause a temperature drop, and thus will not provide the correct temperature.
- Pre-treatment, Denaturation and Hybridization are the key steps in the procedure, if any of these steps in the procedure have not been carried out properly, the results may not be relied upon.

## 1.9 Preparation of supplied reagents in advance

*Pepsin stock solution*:
Dissolve the pepsin digestion reagent in 8 mL distilled or deionised water (upon receipt of the kit). Aliquot in portions of i.e. 1000 µl or 65 µl and store at-20°C. This depends on the expectation of the amount of slides that are subject per test run.

*Pepsin diluent*:
Measure 15 mL of the supplied pepsin diluent (1M HCl) 100x and dilute to 0.01 M HCl with distilled or deionised water.

*Proteolytic work solution:*
Prepare fresh work solution just before use and discard non-used solution:
-Dilute aliquoted proteolytic reagent to 1.25 mg/mL for FFPE (1000 µl pepsin stock in 100 ml pepsin diluent), 100 µg/ml for cytological specimen (60 µl pepsin stock in 75 ml pepsin diluent) and 50 µg/ml for frozen sections (30 µl pepsin stock in 75 ml pepsin diluent) in 0.01 M HCl.
-Pre-heat 0.01 M HCl solution at 37°C, add the required pepsin stock solution just before use and mix.
**Do not re-use**

*TBS buffer:*
Dilute one supplied pouch of the TBS buffer salt in 1 L of distilled water.
**Do not re-use**

## 1.10 Preparation of reagents and materials needed but not supplied

*Dewaxing reagents*
i.e. xylene and ethanol 100%
**Do not re-use**

*Dehydration reagents*
i.e. graded ethanol series (ethanol 70%-96%-100%)

## 1.11 Microscope and accessories

- **Microscope**

A brightfield microscope is needed to validate the *in situ* hybridization results, depending on the application of the end-user.

- **Oculars and objectives**

10X oculars are sufficient, 20X or 40X objectives are usually used for scanning the section. It is recommended to use at least a 63X oil immersion objective for final analysis.

- **Light source**

The light source of regular microscopes is often 50 or 100 W. However a 100 W light source is recommended to obtain optimal results.

- **Immersion oil**

The use of quality immersion oil is recommended; the immersion oil should be suited for use in fluorescence or brightfield microscopy, depending on the application of the end-user


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# Chapter 2 REMBRANDT® DISH Protocol

**- All incubation steps should be performed in a closed incubation chamber/staining jar which contains a liquid (water) creating a saturated moisturized environment. During the incubation steps, evaporation of reagents should be prevented.**
**- Once the hybridization procedure has been started the specimen should not be allowed to dry except for those procedural steps that mention "air-dry".**
**- Allow all reagents to reach the temperatures required for the respective incubation**
**- Work in a fume hood, use forceps and wear protective laboratory clothing and powder-free examination gloves.**

## 2.1 Specimen collection

*Cytological specimen*
Fixation in 70% ethanol is a commonly used for cytological specimen, it preserves the morphology and target DNA. Other fixation methods such as Carnoy's fixative are also applicable.

Sample preparation: Incubate slides in pre-heated proteolytic work solution at 37 °C (100 µg/ml) for 15 minutes. Flush wash in deionised water, followed by dehydration in graded ethanol series (ethanol 70%, 96%, 96%, 100% and 100%), 1 minutes each and air-dry slides for 15 minutes. Continue with proteolytic 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 $cm^3$. 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 38-40°C without any additives and collect sections on bio-adhesive (i.e. organ silane) coated glass slides. Bake the slides at 65°C in a dry air oven for 1 hour. 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 subsequent fresh xylene baths 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 subsequent 100% ethanol (2 x 5 minutes), flush wash in deionised water and continue with proteolytic treatment.


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*Frozen sections*
In general, small pieces of tissue (max. 1 $cm^3$) 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 the proteolytic work solution, flush wash in $dH_2O$ and dehydrate the slides in graded ethanol series. Duration of each soak is 1 minute. Air dry the slides and start with the hybridization 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 Hybridization procedure and post-hybridization washes

*Hybridization*
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 an hotplate at 80°C and incubate 10 minutes for FFPE and 3 minutes cytological specimen and frozen tissue sections (denaturation). Transfer slides into a moist environment and incubate for 16 hours at 37°C (during the hybridization 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).

*Stringency washing*
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. Take the slides out, wipe off excess buffer and dry the edges using a lint-free cloth.
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.


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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.
*Please mind **NOT** to perform the stringency washes (PanWash 50% GC) step on specimens incubated with the positive procedure control oligo probe (pink)!*

## 2.4 Detection and staining procedure

Apply 2-3 drops of HRP or AP-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.

In case of HRP detection: 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 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. 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.

In case of AP detection: 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 NBT/BCIP 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. 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 for HRP-AEC detetion and Nuclear Fast Red for AP-NBT/BCIP detection. Wipe off excess reagent and apply 2-3 drops of counterstain to each specimen. Incubate for 1 minute (longer incubation is possible and will


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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.


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# Chapter 3 Interpretation of results

## 3.1 Guidelines for interpretation

- For specific interpretation guidelines and scoring criteria, see REMBRANDT® DISH detection product specific datasheet.
- For assessment it is important to select representative areas of the specimen.
- Over digestion can lead to RNA loss and affect the presence of RNAs within the specimen.

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.

## 3.2 Quality control

*Invalid results*

- In case the control does not stain adequately, the results of the test slides cannot be accepted. The run should be repeated in order to rule out procedural errors. Please check the trouble shooting section below for further possibilities.
- Poor cell morphology may lead to unacceptable results. In these cases we advise to assess the cell morphology quality in parallel brightfield stained specimen before performing a repeat test.
- If high background signals are present over the cytoplasm obscuring the true signals, the assay should be repeated.
- If hybridization signals are not reliable/identifiable, the assay should be repeated.

For additional requirements, see the product specific datasheet.



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# Chapter 4 Limitations of Procedure

## 4.1 Limitations

- The REMBRANDT® DISH detection assays are solely applicable for the detection of corresponding DNA, as described in the product specific intended use, which may be present in the test 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 DNA probes as supplied with the kit.
- Sample fixation methods should be performed according to the recommended sample preparation methods.
- Many factors can influence the performance of the DISH procedure. Failure in detection can be due to i.e. improper sampling, handling, the time lapse between tissue removal and fixation, the fixation time, processing fixed specimen, the bio-adhesive on the slide, incubation times, pre-treatment procedures, incubation temperatures, freezing, thawing, washing, heating drying, reagent contaminations and interpretation of results.
- The performance of REMBRANDT® DISH detection assays was tested and validated when using the exact procedure as listed in the product specific data sheets, modifications to the procedure may alter the performance characteristics.
- The REMBRANDT® DISH detection assays 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.
- The medical profession should be aware of risks and factors influencing the intensity, the absence or presence of ISH signals which cannot 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, professional and be able to interpret the test results.
- The specific DISH detection assay should only be used for the loci the probe targets.


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*Product in combination with other devices*

The REMBRANDT® DISH detection assays are intended for stand-alone usage. The assay is intended to be used in combination with standard formalin fixed or cytological specimen, 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 formalin fixed or cytological specimen, 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 and other reagents and a microscope is not combined with the device as a product, conformity with the essential requirements is not applicable. Laboratory assay validation should always be established by the end-users.



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# Chapter 5 References

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# Chapter 6 Trouble shooting guide

## 6.1 Introduction

This Trouble Shooting Guide is intended to support you in obtaining optimal results with PanPaths REMBRANDT® DISH detection assays (Duffy et al., 2012) (Arsham et al., 2017).

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 B.V. directly. Since we consider your problem as our problem, we will do our utmost to find a solution.

## 6.2 Cytological specimen

| Problem | Possible causes | Remedies |
|---|---|---|
| Weak or no signals | ■ Sample preparation | → Make sure samples are prepared according to protocol |
| | ■ Proteolytical pre-treatment | → Make sure correct concentration of pre-treatment solutions is used<br>→ Make sure pre-treatments are performed at correct temperatures<br>→ Make sure dehydration steps are performed as recommended<br>→ Make sure that all pre-treatment steps are performed |
| | ■ Hybridization procedure | → Homogenize probe solution prior to applying probe on the section |
| | ■ PanWash temperature and incubation time | → Make sure correct temperatures are maintained and that incubation time of washing step is followed |
| | ■ Coverslips removed with force | → Make sure that slides are soaked and coverslips are not removed with force |
| | ■ Air-dry omitted | → Make sure that when air-dry is recommended, this is done properly |
| | ■ Air bubbles under the coverslip | → Coverslip on a dark surface to visualise if any |
| | ■ Insufficient dehydration | → Prepare fresh dehydration solutions |
| | ■ Photo-damage to the fluorescently labelled probe | → Try to prevent prolonged light exposure and work in a dark environment if applicable |

| | | |
|---|---|---|
| | ■ No target sequence present | → Use appropriate controls |
| | ■ Microscope and accessories | → Check filter sets<br>→ Check lamps<br>→ Check running hours of lamp<br>→ Check lamp alignment<br>→ Check and clean lenses and mirror<br>→ Call microscope technical service<br>→ Use recommended objectives and filters as described in the product specific datasheet |
| Non-specific background staining and signal variation | ■ Cellular debris in cell preparation | → Perform additional wash steps with fresh fixative to remove debris |
| | ■ Denaturation temperature too high | → Make sure temperature is 80 °C |
| | ■ Denaturation step too long | → Denature no longer than 5 minutes |
| | ■ Washing temperature | → Make sure correct temperatures are maintained<br>→ Make sure pH is adjusted to 7.4 |
| | ■ Proteolytic pre-treatment too strong | → Make sure that the concentration of pepsin is as described in the product specific datasheet and do not exceed the incubation time in proteolytic work solution |

## 6.3 Paraffin embedded tissue sections

| Problem | Possible causes | Remedies |
|---|---|---|
| No section left on the slides or morphology detected | ■ 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 2-4 µm |
| | ■ Wrong (side of) glass slide used | → Use only plus coated glass slides |
| | ■ Pepsin concentration too high | → Make sure correct concentration of pepsin is used. |
| | ■ Proteolytic pre-treatment step too long | → Reduce time of proteolytic pre-treatment step |


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| | | |
|---|---|---|
| | ■ Denaturation | → Make sure temperature is 80°C<br>→ Denature no longer than 10 minutes |
| | ■ Coverslips removed with force | → Make sure that slides are soaked and coverslips are not removed with force |
| | ■ Post-fix solution omitted | → Make sure post-fix incubation is performed |
| | ■ Air-dry omitted | → Make sure that when air-dry is recommended, this is done properly |
| Weak or no signals | ■ Tissue fixation | → Only use buffered formalin fixative and check fixation time (prevent over-fixation) |
| | ■ Deparaffinisation | → Renew dewax series |
| | ■ Pre-treatment | → Make sure correct concentration of pre-treatment solutions is used<br>→ Make sure pre-treatments are performed at correct temperatures<br>→ Make sure dehydration steps are performed as recommended<br>→ Make sure that all pre-treatment steps are performed |
| | ■ Denaturation | → Make sure temperature is 80 °C |
| | ■ Hybridization procedure | → Homogenize probe solution prior to applying probe on the section |
| | ■ PanWash temperature and incubation time | → Make sure correct temperatures are maintained and that incubation time of washing step is followed |
| | ■ Air bubbles during denaturation and hybridization | → Make sure that when covering section with cover slip, air bubbles are not present |
| | ■ Insufficient amount of probe used | → Make sure that sufficient amount of probe is used to cover the section |
| | ■ No target sequence present | → Use appropriate controls |
| | ■ Photo-damage to the fluorescently labelled probe | → Try to prevent prolonged light exposure and work in a dark environment if applicable |
| | ■ Microscope and accessories | → Check filter sets<br>→ Check lamps<br>→ Check running hours of lamp |


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| | | |
|---|---|---|
| | | → Check lamp alignment<br>→ Check and clean lenses and mirror<br>→ Call microscope technical service |
| Non-specific background staining and signal variation | ■ Tissue section too thick | → Optimal thickness of the tissue is 2-4 µm |
| | ■ Tissue crumbled | → Make sure tissue is stretched completely |
| | ■ Deparaffinisation | → Dewax series |
| | ■ Denaturation temperature too high | → Make sure temperature is 80 °C |
| | ■ Hybridization: uneven distribution of probe due to air bubbles/uneven section | → Repeat procedure on new section |
| | ■ Denaturation step too long | → Denature no longer than 10 minutes |
| | ■ Drying out of the section | → Incubate all procedure steps in a moisturised environment; prevent evaporation |
| | ■ Washing temperature | → Make sure correct temperatures are maintained<br>→ Make sure pH is adjusted to 7.4 |

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.


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## Immaterial property information

REMBRANDT® is a registered trade name of PanPath B.V., Budel, The Netherlands.

Purchase does not include the right to exploit this product commercially and any commercial use without the explicit authorization of PanPath BV is prohibited.



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# REMBRANDT®

*In situ* Hybridization and Detection

## *Universal DISH detection assays*-*v4.2023*

| Universal DISH kits | | |
|---|---|---|
| **Label/Detection** | **Product number** | **# assays** |
| BIO-HRP | A001K.0101 | 10-100 T |
| DIG-HRP | A001K.9901 | 10-100 T |
| BIO-AP | A001K.0105 | 10-100 T |
| DIG-AP | A001K.9905 | 10-100 T |

**RUO**


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# Contents





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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* Hybridization (ISH) technique in cytological specimen or FFPE tissue sections. In extensive tests and field application, the REMBRANDT® Universal DISH kits were proven to be very robust, and ISH results were highly reproducible. For the specific intended use of a REMBRANDT® Universal DISH detection assay, please see the product-specific datasheet.

## 1.2 The DISH principle

DISH enables the detection of specific DNA sequences in histological and cytological specimens, without losing the often very essential morphological details. The principle of DISH is based on a "reaction" (= hybridization) between a specifically conjugated oligonucleotide sequence (= probe) and an DNA sequence present in the sample (= target). In case of matching sequences, a hybrid between the probe and target will be formed. The formed hybrids can easily be visualized after detection with corresponding antibodies by brightfield microscopy. The REMBRANDT® Universal DISH probes are conjugated to biotin or digoxigenin; the conjugate used in the kits depend on specific probe(s) in the kit (Arsham et al., 2017).

## 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. Additional control slides and probes are available from PanPath B.V.; please contact your local supplier.

## 1.4 Contents of a REMBRANDT® Universal DISH detection assay

| Item label description | | Item (cap) colour | Items contents description | Item amount |
|---|---|---|---|---|
| DIGEST | PEPSIN POW | Transparant vial | Pepsin digestion reagents | 1 gram |
| DIGEST | PEPSIN DIL | Transparent vial | Pepsin diluent | 15 ml |
| PROBE | + \| ..[1] DISH | Pink cap | DISH positive control oligo probe (BIO or DIG) | 1 ml |
| PROBE | - \| ..[1] DISH | Green cap | DISH negative control oligo probe (BIO or DIG) | 1 ml |
| CONJ | .....[1] HRP or AP | Red cap | HRP or AP conjugated anti BIO or anti DIG | 15 ml |
| PANWASH | GC% ...50% | White cap | PanWash 50% GC concent | 2x 15 ml |
| SUBS | AEC[2] | Blue cap | AEC substrate | 2 ml |
| BUFF | AEC or NBT/BCIP [1] | Blue cap | AEC buffer or NBT/BCIP substrate/buffer | 15 ml |
| COUNT | MG or FR [1] | Orange cap | Methyl green or Fast red counterstain | 15 ml |


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| | | | | |
|---|---|---|---|---|
| WASH | TBS | White pouches | TBS buffer salt | 2 pcs |
| SUPPORT | GL SLIDES | White box A | Coated glass slides | 50 pcs |
| SUPPORT | COVERSLIPS | White box B | Coverslips | 100 pcs |
| CONTROL | QC SLIDES | Transparant box | Positive control slides | 3 pcs |

1 Depends on kit specification

## 1.5 Materials required but not included

- Xylene for dewaxing paraffin sections
- Fixative for cytological specimens
- Distilled or deionised water
- Ethanol series - 100% Ethanol
  - 96% Ethanol
  - 70% Ethanol
- Water based mounting medium
- Pipettes and tips to deliver 10-1000 µL
- Immersion oil for brightfield microscopy
- Timer(s)
- Microwave (optional)
- Mircoscope and accessories
- Oven(s), heating block(s) and water baths
- Incubation chamber
- Mixing device i.e. vortex
- Magnetic stirrer
- (Surface) thermometer; calibrated
- pH meter and calibrators
- Coverslips
- Slides

## 1.6 Storage and shelf life

– Store kit and its contents at 2-8°C.
– Store the dissolved and aliquoted reagents 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 expiry date printed on the box.

## 1.7 Safety precautions

– If reagents come into contact with skin or eyes, rinse with large volumes of clean water.
– Never pipette solutions by mouth.
– All hazardous materials should be disposed of according to national guidelines.
– A material safety data sheet is available on request

## 1.8 Performance precautions

– Read all instructions before processing any assay.
– Store all reagents as recommended.
– ***DO NOT*** use reagents beyond their expiry date.
– Allow all components to warm up to room temperature (20-25°C) before use.



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- Check all ready to use solutions for precipitates and other contaminations before use; if contaminations are present, the solution should be disposed of, and a new solution should be prepared.
- PanWash 4 concentrated solution may form crystals when stored at low temperatures. If heating does not dissolve the crystals, the solution should be disposed of and a new solution should be prepared.
- Homogenize all solutions before use.
- Homogenize probe solution and spin briefly before use.
- ***DO NOT*** substitute a reagent with one from another manufacturer.
- Use treated glass slides, preferably REMBRANDT® Adhesive coated glass slides (Z000S.0002, PanPath B.V.).
- ***DO NOT*** re-use prepared, ready to use digestion reagent; dispose of residuals.
- With every new procedure, check the temperatures of pre-treatment solutions, denaturation device used, incubation device used and PanWash solution by using a **calibrated** thermometer.
- Do not incubate more than 5 specimens at the same time in pre-set temperature baths/devices. Placing more than 5 specimens in such a device will cause a temperature drop, and thus will not provide the correct temperature.
- Pre-treatment, Denaturation and Hybridization are the key steps in the procedure, if any of these steps in the procedure have not been carried out properly, the results may not be relied upon.

## 1.9 Preparation of supplied reagents in advance

*Pepsin stock solution*:
Dissolve the pepsin digestion reagent in 8 mL distilled or deionised water (upon receipt of the kit). Aliquot in portions of i.e. 1000 µl or 65 µl and store at-20°C. This depends on the expectation of the amount of slides that are subject per test run.

*Pepsin diluent*:
Measure 15 mL of the supplied pepsin diluent (1M HCl) 100x and dilute to 0.01 M HCl with distilled or deionised water.

*Proteolytic work solution:*
Prepare fresh work solution just before use and discard non-used solution:
-Dilute aliquoted proteolytic reagent to 1.25 mg/mL for FFPE (1000 µl pepsin stock in 100 ml pepsin diluent), 100 µg/ml for cytological specimen (60 µl pepsin stock in 75 ml pepsin diluent) and 50 µg/ml for frozen sections (30 µl pepsin stock in 75 ml pepsin diluent) in 0.01 M HCl.
-Pre-heat 0.01 M HCl solution at 37°C, add the required pepsin stock solution just before use and mix.
**Do not re-use**

*TBS buffer:*
Dilute one supplied pouch of the TBS buffer salt in 1 L of distilled water.
**Do not re-use**

## 1.10 Preparation of reagents and materials needed but not supplied

*Dewaxing reagents*
i.e. xylene and ethanol 100%
**Do not re-use**

*Dehydration reagents*
i.e. graded ethanol series (ethanol 70%-96%-100%)

## 1.11 Microscope and accessories

- **Microscope**

A brightfield microscope is needed to validate the *in situ* hybridization results, depending on the application of the end-user.

- **Oculars and objectives**

10X oculars are sufficient, 20X or 40X objectives are usually used for scanning the section. It is recommended to use at least a 63X oil immersion objective for final analysis.

- **Light source**

The light source of regular microscopes is often 50 or 100 W. However a 100 W light source is recommended to obtain optimal results.

- **Immersion oil**

The use of quality immersion oil is recommended; the immersion oil should be suited for use in fluorescence or brightfield microscopy, depending on the application of the end-user


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# Chapter 2 REMBRANDT® Universal DISH Protocol

**- All incubation steps should be performed in a closed incubation chamber/staining jar which contains a liquid (water) creating a saturated moisturized environment. During the incubation steps, evaporation of reagents should be prevented.**
**- Once the hybridization procedure has been started the specimen should not be allowed to dry except for those procedural steps that mention "air-dry".**
**- Allow all reagents to reach the temperatures required for the respective incubation**
**- Work in a fume hood, use forceps and wear protective laboratory clothing and powder-free examination gloves.**

## 2.1 Specimen collection

*Cytological specimen*
Fixation in 70% ethanol is a commonly used for cytological specimen, it preserves the morphology and target DNA. Other fixation methods such as Carnoy's fixative are also applicable.

Sample preparation: Incubate slides in pre-heated proteolytic work solution at 37 °C (100 µg/ml) for 15 minutes. Flush wash in deionised water, followed by dehydration in graded ethanol series (ethanol 70%, 96%, 96%, 100% and 100%), 1 minutes each and air-dry slides for 15 minutes. Continue with proteolytic 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 $cm^3$. 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 38-40°C without any additives and collect sections on bio-adhesive (i.e. organ silane) coated glass slides. Bake the slides at 65°C in a dry air oven for 1 hour. 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 subsequent fresh xylene baths 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 subsequent 100% ethanol (2 x 5 minutes), flush wash in deionised water and continue with proteolytic treatment.


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*Frozen sections*
In general, small pieces of tissue (max. 1 $cm^3$) 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 the proteolytic work solution, flush wash in $dH_2O$ and dehydrate the slides in graded ethanol series. Duration of each soak is 1 minute. Air dry the slides and start with the hybridization 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 Hybridization procedure and post-hybridization washes

*Hybridization*
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 an hotplate at 80°C and incubate 10 minutes for FFPE and 3 minutes cytological specimen and frozen tissue sections (denaturation). Transfer slides into a moist environment and incubate for 16 hours at 37°C (during the hybridization 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).

*Stringency washing*
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. Take the slides out, wipe off excess buffer and dry the edges using a lint-free cloth.
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.


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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.
*Please mind **NOT** to perform the stringency washes (PanWash 50% GC) step on specimens incubated with the positive procedure control oligo probe (pink)!*

## 2.4 Detection and staining procedure

Apply 2-3 drops of HRP or AP-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.

In case of HRP detection: 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 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. 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.

In case of AP detection: 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 NBT/BCIP 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. 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 for HRP-AEC detetion and Nuclear Fast Red for AP-NBT/BCIP detection. Wipe off excess reagent and apply 2-3


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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.


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# Chapter 3 Interpretation of results

## 3.1 Guidelines for interpretation

- For specific interpretation guidelines and scoring criteria, see REMBRANDT® Universal DISH detection product specific datasheet.
- For assessment it is important to select representative areas of the specimen.
- Over digestion can lead to RNA loss and affect the presence of RNAs within the specimen.

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.

## 3.2 Quality control

*Invalid results*

- In case the control does not stain adequately, the results of the test slides cannot be accepted. The run should be repeated in order to rule out procedural errors. Please check the trouble shooting section below for further possibilities.
- Poor cell morphology may lead to unacceptable results. In these cases we advise to assess the cell morphology quality in parallel brightfield stained specimen before performing a repeat test.
- If high background signals are present over the cytoplasm obscuring the true signals, the assay should be repeated.
- If hybridization signals are not reliable/identifiable, the assay should be repeated.

For additional requirements, see the product specific datasheet.



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# Chapter 4 Limitations of Procedure

## 4.1 Limitations

- The REMBRANDT® Universal DISH detection assays are solely applicable for the detection of corresponding RNA, as described in the product specific intended use, which may be present in the test 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 DNA probes as supplied with the kit.
- Sample fixation methods should be performed according to the recommended sample preparation methods.
- Many factors can influence the performance of the DISH procedure. Failure in detection can be due to i.e. improper sampling, handling, the time lapse between tissue removal and fixation, the fixation time, processing fixed specimen, the bio-adhesive on the slide, incubation times, pre-treatment procedures, incubation temperatures, freezing, thawing, washing, heating drying, reagent contaminations and interpretation of results.
- The performance of REMBRANDT® Universal DISH detection assays was tested and validated when using the exact procedure as listed in the product specific data sheets, modifications to the procedure may alter the performance characteristics.
- The REMBRANDT® ISH detection assays 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.
- The medical profession should be aware of risks and factors influencing the intensity, the absence or presence of ISH signals which cannot 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, professional and be able to interpret the test results.
- The specific DISH detection assay should only be used for the loci the probe targets.

*Product in combination with other devices*

The REMBRANDT® DISH detection assays are intended for stand-alone usage. The assay is intended to be used in combination with standard formalin fixed or cytological specimen, 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 formalin fixed or cytological specimen, 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 and other reagents and a microscope is not combined with the device as a product, conformity with the essential requirements is not applicable. Laboratory assay validation should always be established by the end-users.


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# Chapter 5 References

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# Chapter 6 Trouble shooting guide

## 6.1 Introduction

This Trouble Shooting Guide is intended to support you in obtaining optimal results with PanPaths REMBRANDT® DISH detection assays (Duffy et al., 2012) (Arsham et al., 2017).

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 B.V. directly. Since we consider your problem as our problem, we will do our utmost to find a solution.

## 6.2 Cytological specimen

| Problem | Possible causes | Remedies |
|---|---|---|
| Weak or no signals | ■ Sample preparation | → Make sure samples are prepared according to protocol |
| | ■ Proteolytical pre-treatment | → Make sure correct concentration of pre-treatment solutions is used<br>→ Make sure pre-treatments are performed at correct temperatures<br>→ Make sure dehydration steps are performed as recommended<br>→ Make sure that all pre-treatment steps are performed |
| | ■ Hybridization procedure | → Homogenize probe solution prior to applying probe on the section |
| | ■ PanWash temperature and incubation time | → Make sure correct temperatures are maintained and that incubation time of washing step is followed |
| | ■ Coverslips removed with force | → Make sure that slides are soaked and coverslips are not removed with force |
| | ■ Air-dry omitted | → Make sure that when air-dry is recommended, this is done properly |
| | ■ Air bubbles under the coverslip | → Coverslip on a dark surface to visualise if any |
| | ■ Insufficient dehydration | → Prepare fresh dehydration solutions |
| | ■ Photo-damage to the fluorescently labelled probe | → Try to prevent prolonged light exposure and work in a dark environment if applicable |


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| | | |
|---|---|---|
| | ■ No target sequence present | → Use appropriate controls |
| | ■ Microscope and accessories | → Check filter sets<br>→ Check lamps<br>→ Check running hours of lamp<br>→ Check lamp alignment<br>→ Check and clean lenses and mirror<br>→ Call microscope technical service<br>→ Use recommended objectives and filters as described in the product specific datasheet |
| Non-specific background staining and signal variation | ■ Cellular debris in cell preparation | → Perform additional wash steps with fresh fixative to remove debris |
| | ■ Denaturation temperature too high | → Make sure temperature is 80 °C |
| | ■ Denaturation step too long | → Denature no longer than 5 minutes |
| | ■ Washing temperature | → Make sure correct temperatures are maintained<br>→ Make sure pH is adjusted to 7.4 |
| | ■ Proteolytic pre-treatment too strong | → Make sure that the concentration of pepsin is as described in the product specific datasheet and do not exceed the incubation time in proteolytic work solution |

## 6.3 Paraffin embedded tissue sections

| Problem | Possible causes | Remedies |
|---|---|---|
| No section left on the slides or morphology detected | ■ 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 2-4 µm |
| | ■ Wrong (side of) glass slide used | → Use only plus coated glass slides |
| | ■ Pepsin concentration too high | → Make sure correct concentration of pepsin is used. |

| | | |
|---|---|---|
| | ■ Proteolytic pre-treatment step too long | → Reduce time of proteolytic pre-treatment step |
| | ■ Denaturation | → Make sure temperature is 80°C<br>→ Denature no longer than 10 minutes |
| | ■ Coverslips removed with force | → Make sure that slides are soaked and coverslips are not removed with force |
| | ■ Post-fix solution omitted | → Make sure post-fix incubation is performed |
| | ■ Air-dry omitted | → Make sure that when air-dry is recommended, this is done properly |
| Weak or no signals | ■ Tissue fixation | → Only use buffered formalin fixative and check fixation time (prevent over-fixation) |
| | ■ Deparaffinisation | → Renew dewax series |
| | ■ Pre-treatment | → Make sure correct concentration of pre-treatment solutions is used<br>→ Make sure pre-treatments are performed at correct temperatures<br>→ Make sure dehydration steps are performed as recommended<br>→ Make sure that all pre-treatment steps are performed |
| | ■ Denaturation | → Make sure temperature is 80 °C |
| | ■ Hybridization procedure | → Homogenize probe solution prior to applying probe on the section |
| | ■ PanWash temperature and incubation time | → Make sure correct temperatures are maintained and that incubation time of washing step is followed |
| | ■ Air bubbles during denaturation and hybridization | → Make sure that when covering section with cover slip, air bubbles are not present |
| | ■ Insufficient amount of probe used | → Make sure that sufficient amount of probe is used to cover the section |
| | ■ No target sequence present | → Use appropriate controls |
| | ■ Photo-damage to the fluorescently labelled probe | → Try to prevent prolonged light exposure and work in a dark environment if applicable |

| | | |
|---|---|---|
| | ■ Microscope and accessories | → Check filter sets<br>→ Check lamps<br>→ Check running hours of lamp<br>→ Check lamp alignment<br>→ Check and clean lenses and mirror<br>→ Call microscope technical service |
| Non-specific background staining and signal variation | ■ Tissue section too thick | → Optimal thickness of the tissue is 2-4 µm |
| | ■ Tissue crumbled | → Make sure tissue is stretched completely |
| | ■ Deparaffinisation | → Dewax series |
| | ■ Denaturation temperature too high | → Make sure temperature is 80 °C |
| | ■ Hybridization: uneven distribution of probe due to air bubbles/uneven section | → Repeat procedure on new section |
| | ■ Denaturation step too long | → Denature no longer than 10 minutes |
| | ■ Drying out of the section | → Incubate all procedure steps in a moisturised environment; prevent evaporation |
| | ■ Washing temperature | → Make sure correct temperatures are maintained<br>→ Make sure pH is adjusted to 7.4 |

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.


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## Immaterial property information

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