CE IVD PanPath

Directive 98/79/EC

REMBRANDT®

In situ Hybridisation and Detection

FISH detection assays-*v6.2023*

FISH kit		
Label	**Product number**	**# assays**
Green	CxxxK.2000.05	5 T
Green	CxxxK.2000.10	10 T
Orange	CxxxK.3000.05	5 T
Orange	CxxxK.3000.10	10 T
Green and orange	CxxxK.2030.05	5 T
Green and orange	CxxxK.2030.10	10 T
Green and orange	C801K.5206	20 T

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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 fluorescence *in situ* Hybridisation (FISH) technique in cytological specimen or FFPE tissue sections. The REMBRANDT® kits are to be used to assess the specific status of the target for which the probe is designed by Fluorescence *in situ* hybridisation (FISH) in cytological specimen or FFPE tissue sections. In extensive tests and field application the REMBRANDT® FISH kits were proven to be very robust, and FISH results were highly reproducible. For the specific intended use of a REMBRANDT® FISH detection assay, please see the product specific datasheet.

1.2 The FISH principle

FISH 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 FISH 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-specific hybrids are washed out by the stringency wash procedure (PanWash 4). The formed hybrids can easily be visualised via fluorescent microscopy. The REMBRANDT® probes are labelled with fluorochromes; the fluorochromes used in the kits depend on specific probe(s) in the kit (Arsham et al., 2017).

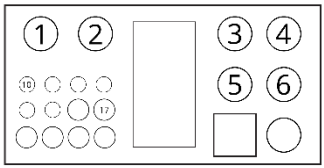
1.3 Controls

Use of controls is an essential part of the routine. To ensure that the FISH 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® FISH detection assay

Application: cytological specimen

Contents REMBRANDT® FISH detection assays



REF XX-YY

Pos.	Contents	Product label		Vol.		REF
1	Pepsin diluent	DIGEST	PEPSIN DIL	15	ml.	R018R.0000
2	Pepsin powder	DIGEST	PEPSIN POW	1	gr.	R011R.0000
3	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
4	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
5	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
6	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
10	Product specific probe	PROBE	SPECIFIC PROBE	75 150	µl µl	XXXXX.YYYY
17	Mounting medium fluorescence	SUPPORT	MOUNT FLU	1	ml.	Z000R.0050

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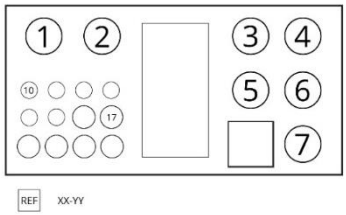
Application: FFPE tissue sections

Contents REMBRANDT® FISH detection assays



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4	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
5	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
6	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
7	Pre-treatment buffer	PRE-TREAT	Na-Citrate 100x	15	ml.	R026R.0000
10	Product specific probe	PROBE	SPECIFIC PROBE	75 150	µl µl	XXXXX.YYYY
17	Mounting medium fluorescence	SUPPORT	MOUNT FLU	1	ml.	Z000R.0050

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
- HCl 0.01 M
- Phosphate buffered saline (PBS); pH 7.2-7.4
- Pipettes and tips to deliver 10-1000 µL
- Immersion oil for fluorescence microscopy
- Timer(s)
- Fluorescence microscope 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
- Microwave (optional)

1.6 Single product catalogue number, storage and shelf life

REMBRANDT® probe mix; 1 vial		**Catalogue No**	CXXXP.XXXX
Quantity	: 75 or 150 µl; 5 or 10 assays/kit	**Storage**	2 – 8°C
Composition	: specific probe DNA fluorescently labelled, ready to use in hybridisation buffer.		

Pepsin digestion reagent; 1 vial		**Catalogue No**	R011R.0000
Quantity	: 1 gr; powder to be dissolved in 8 mL deionised water, to be aliquoted in portions of e.g. 1200 µl or 600 µl depending on the amount of slides per test run	**Storage** powder dissolved solution	 2 – 25°C -20°C
Composition	: Pepsin powder		

Pepsin diluent; 1 vial		**Catalogue No**	R018R.0000
Quantity	: 15 mL 1M to be diluted 100x with deionised water	**Storage**	

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	to 0.01 M HCl	1 M HCl 0.01 M HCl	2 – 25°C 2 – 25°C
Composition	: HCl, 1M		

Concentrated PanWash 4, 25x SSC; 4 vials		**Catalogue No**	R025R.0000
Quantity	: 15 mL concentrated solution to be diluted 12.5x (= 2x SSC) with deionised water and confirm (and adjust if needed) to pH 7.4	**Storage** conc. solution diluted solution	 2 – 25°C ambient temp.
Composition	: Sodium chloride, sodium citrate and surfactant		

Concentrated Pre-treatment buffer, 100x Na-Citrate; 1 vial (optional)		**Catalogue No**	R025R.0000
Quantity	: 15 mL concentrated solution to be diluted 100x (= 1x Na-Citrate) with deionised water and confirm (and adjust if needed) pH 6.0	**Storage** conc. solution diluted solution	 2 – 25°C ambient temp.
Composition	: Natrium citrate		

Mounting medium; 1 vial		**Catalogue No**	Z000R.0050
Quantity	: 2 mL	**Storage**	2 – 8°C
Composition	: DAPI (4,6-diamidino-2-phenylindole), DABCO (diazabicyclo-2.2.2- octane) in TRIS buffer and glycerol		

– Store kit and its contents at 2-8°C.
– Store the dissolved and aliquoted reagents at recommended temperatures (see above).
– When used and stored as indicated, the kit is stable until the expiry date printed on the box.

1.7 Safety precautions

– The hybridisation buffer in which the probes are mixed contains formamide, which is considered as a teratogen; contact with skin and mucous membranes should be avoided.
– The Mounting medium contains DAPI (4,6-diamino-2-phenylindole) and 1,4-phenylenediamine which is a possible respiratory and dermal sensitizer; contact with skin and mucous membranes should be avoided.
– 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 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.
- Homogenise all solutions before use.
- Homogenise 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 4 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 Hybridisation 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.
- Fluorochromes are light sensitive; therefore, all steps that do not require manipulation should be performed under exclusion of light.

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

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PanWash 4:
- Dilute a vial of the supplied 15 mL PanWash 4, 25x SSC to **2x SSC** with 172.5 mL deionised or distilled water to a total of 187.5 ml (concentration is 2x SSC).
- Dilute 0.8 mL of a vial of the supplied PanWash 4, 25x SSC to **0.1x** SSC with 199.2 mL deionised or distilled water to a total of 200 ml (concentration is 0.1x SSC).

Before use:
a) Fill staining jars with SSC solution.
b) Pre-heat 2x SSC at 42°C and 0.1x SSC at 61°C (if applicable)
Do not re-use

Pre-treatment buffer (included depending assay type; see product specific datasheet):
Dilute the supplied 15 mL Pre-treatment buffer, 100x Na-Citrate to 1x Na-Citrate with 1485 mL deionised or 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%)

PBS (phosphate buffered saline)/0.05% Tween
- Deionised water:
  950 ml
- Sodium chloride (NaCL):
  8 g
- Potassium chloride (KCl):
  0.2 g
- di-Sodium hydrogen phosphate di-hydrate ($Na_2HPO_4.2H_2O$):
  1.78 g
- Potassium di-hydrogen phosphate (KH_2PO_4):
  0.24 g
- Tween® 20
  0.5 ml

Adjust pH to 7.4 +/- 0.2 and adjust to 1000 mL with deionised water
Do not re-use

1.11 Microscope and accessories

– **Microscope**

A fluorescence microscope is needed to validate the *in situ* hybridisation results.

– **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.

– **Filters**

Most, if not all microscope manufactures, can deliver the needed single and multi-band pass filters. The single and multi-band pass filters needed for the REMBRANDT® FISH detection assays are:

Filter set for DAPI	
excitation	G 365 nm
beam splitter	FT 395 nm
emission	LP 420 nm

Filter set for green fluorochrome	
Excitation	BP 450 nm – 490 nm
beam splitter	FT 510 nm
emission	BP 515 nm – 565 nm

Filter set for orange fluorochrome	
Excitation	BP 546 nm /12
beam splitter	FT 580
emission	LP 590 nm

Triple band filter set (DAPI / FITC / TRITC)

– **Immersion oil**

The use of quality immersion oil is recommended; the immersion oil should have low auto-fluorescence and be suited for use in fluorescence microscopy.

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Chapter 2 REMBRANDT® FISH Protocol

- All incubation steps should be performed in a closed (dark) incubation chamber/staining jar 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 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.

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 FISH, 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 pre-treatment.

2.2 Pre-treatment of specimen

Cytological specimen
- 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 minute each and air-dry slides for 15 minutes.

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Paraffin embedded tissue sections

For making the DNA accessible for the probes and to obtain reliable and reproducible results, pre-treatment procedures are necessary.

- Place slides in jar filled with pre-treatment buffer (R026R.0000), place the jar in a microwave set at i.e. 900W and incubate up until boiling, subsequently reset microwave at 180W and proceed with the incubation for 10 minutes followed by a 20 minute cool down period at room temperature; all in the same solution. Flush wash slides in deionised water at room temperature.
- Incubate slides in pre-heated proteolytic work solution at 37 °C (1.25 mg/ml) for 15 minutes.
- Flush wash in deionised water, followed by dehydration in graded ethanol series (ethanol 70%, 96%, 96%, 100% and 100%), 1 minute each and air-dry slides for 15 minutes.

Do not treat more than 5 slides at the same time, because the temperature of the pre-heated solutions may drop dramatically, thus causing incomplete pre-treatment. Additionally, allow the slides to air-dry as recommended; otherwise sections will be lost.

2.3 Hybridisation procedure and post-hybridization washes

Denaturation and Hybridisation

- Homogenize probe solution and spin briefly. Apply 10-15 µl of probe solution to each specimen. Cover all specimens with a cover slip (avoid air bubbles).
- Place slides on an 80°C hotplate or other heating device and incubate for 3 minutes in case of cytological specimen and 10 minutes in case of FFPE tissue sections (denaturation).

Work in a pre-set order to ensure that all slides have been incubated at 80°C for the exact same time! Do not denature more than 5 slides at the same time, because the temperature of the heating device may drop dramatically, thus causing incomplete denaturation.

- Transfer slides into a moist environment and incubate in the dark for 16 hours at 37°C.

Differentiation (stringent wash) and rinsing

- Remove coverslips by submerging the slides in PBS at room temperature. Soak the slides until the coverslips fall off.
- Incubate slides in pre-heated PanWash 4, **buffer concentration and incubation temperature differ per specific product** (see product specific datasheet).
- Incubate slides in PBS at room temperature for 1 minute.
- Dehydrate in graded ethanol series. Air-dry slides for 15 minutes (in the dark).

Do not incubate more than 5 slides at the same time in PanWash 4 (2x SSC and 0.1x SSC), because the temperature of PanWash 4 may drop dramatically, causing wrong stringency conditions.

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Coverslipping

Mount slides by applying 1-2 drops of the supplied mounting medium (Z000R.0050) and coverslip.

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Chapter 3 Interpretation of results

3.1 Guidelines for interpretation

- For specific interpretation guidelines and scoring criteria, see REMBRANDT® FISH detection product specific datasheet.
- For assessment it is important to select representative areas of the specimen.
- Over digestion can lead to DNA loss and affect the gene signal numbers in individual nuclei. Score only nuclei that have relatively intact nuclear borders.

3.2 Quality control

Positive control

It is advised to run a positive and negative control simultaneously with each FISH assay. As a positive control, one may also use validated in-house specimen. If the controls fail to demonstrate the expected staining, the result on the test specimen must be validated as invalid.

Assay requirements

- Noise-to-signal percentage
  A FISH probe is detected via fluorescent microscopy. The probe will emit a fluorescent signal. However, a fluorescent background signal can also be observed in the cells. Therefore, the noise-to-signal cut-off needs to be determined for FISH probes. If the fluorescent signal in the background is too close to the actual probe signal, the probe is not useable in diagnostics. For good signal interpretation, the noise-to-signal percentage should be ≤40%.
- Hybridization efficiency
  Signals may only be interpreted if the probe signals are visualized in ≥98% of the cells.

For additional requirements, see the product specific datasheet.

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 noise-to-signal percentage. 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 nuclear borders are lost or persistent green or red auto-fluorescence masks true signals, the enzymatic digestion was not optimal and the test should be repeated.
- If hybridisation signals are not reliable/identifiable, the assay should be repeated.

For additional requirements, see the product specific datasheet.

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Chapter 4 Performance characteristics

4.1 Analytical performance

Analytical performance studies were performed according to Mascarello et al., 2011 and Arsham et al., 2017 in accordance with the IVD DIRECTIVE 98/79/EC.

Analytical specificity

The specificity of all REMBRANDT® FISH probes were verified by sequencing and assessed on normal lymphocyte metaphase spreads. Metaphase preparations were obtained from different healthy donors and prepared according routine procedure. Prior to FISH, the chromosomes were stained using the GTG-banding method identifying the chromosome/gene of the probe of interest. For details regarding the analytical performance, see the product specific datasheets .

Analytical sensitivity

The sensitivity of all REMBRANDT® FISH probes were assessed by determination of several factors which influence probe sensitivity. For each REMBRANDT® FISH probe the normal cut-off, noise-to-signal cut-off and the hybridization efficiency were determined. For details regarding the analytical performance, see the product specific datasheets .

Precision

The precision of the REMBRANDT® FISH probes were assessed by determination of the precision at three different levels: repeatability (intra-assay), intermediate precision (intra-lab), and reproducibility. For details regarding the precision, data of specific REMBRANDT® FISH products is available upon request.

Stability

The stability of the REMBRANDT® FISH probes were assessed by performance of classical stability studies according to the EP25AE Evaluation of Stability of *In Vitro* Diagnostic Reagents, 1st Edition (Wayne, 2009). To determine the stability, the shelf life, in-use and shipping stability were assessed. For details regarding the stability, data of specific REMBRANDT® FISH products is available upon request.

4.2 Clinical performance

Clinical performance depends strongly on the intended use of the assay. Clinical performance has been evaluated for certain REMBRANDT® products detecting a specific disease, condition or result in a diagnosis. For details regarding clinical performance, please contact info@panpath.nl

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Chapter 5 Limitations of Procedure

5.1 Limitations

- The REMBRANDT® FISH 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 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 FISH procedure. Failure in detection can be due to i.e. improper sampling, handling, the time lapse between tissue sample 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® FISH 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 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 clinical characteristics and phenotypic features. Negative results therefore do not rule out any possibility of a positive specimen.
- The REMBRANDT® FISH 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.
- Therapeutic considerations based on the result of this test alone should not been taken. Positive results should be verified by other traditional diagnostic methods such as but not limited to clinical history, symptoms, as well as clinical characteristics and phenotypic features.
- The medical profession should be aware of risks and factors influencing the intensity, the absence or presence of FISH 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 FISH detection assay should only be used for the loci the probe targets.

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Product in combination with other devices

The REMBRANDT® FISH 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 6 References

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Chapter 7 Trouble shooting guide

7.1 Introduction

This Trouble Shooting Guide is intended to support you in obtaining optimal results with PanPaths REMBRANDT® FISH 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.

7.2 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 → Make sure pre-treatments are performed at correct temperatures → Make sure dehydration steps are performed as recommended → Make sure that all pre-treatment steps are performed
	■ Hybridisation procedure	→ Homogenize probe solution prior to applying probe on the section
	■ PanWash 4 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

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	■ Microscope and accessories	→ Check filter sets → Check lamps → Check running hours of lamp → Check lamp alignment → Check and clean lenses and mirror → Call microscope technical service → 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 → 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

7.3 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 → Denature no longer than 10 minutes

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	■ 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 → Make sure pre-treatments are performed at correct temperatures → Make sure dehydration steps are performed as recommended → Make sure that all pre-treatment steps are performed
	■ Denaturation	→ Make sure temperature is 80 °C
	■ Hybridisation procedure	→ Homogenize probe solution prior to applying probe on the section
	■ PanWash 4 temperature and incubation time	→ Make sure correct temperatures are maintained and that incubation time of washing step is followed
	■ Air bubbles during denaturation and hybridisation	→ 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 → Check lamps → Check running hours of lamp → Check lamp alignment → Check and clean lenses and mirror → Call microscope technical service

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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
	■ Hybridisation: 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 → 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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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 Hybridisation and Detection

FISH detection assays-*v7.2023*

FISH kit		
Label	**Product number**	**# assays**
Green	CxxxK.2000.05 CxxxK.2000.10	5 T 10 T
Orange	CxxxK.3000.05 CxxxK.3000.10	5 T 10 T
Green and orange	CxxxK.2030.05 CxxxK.2030.10	5 T 10 T
Green, Orange and Blue	CxxxK.203040.05 CxxxK.203040.10	5 T 10 T
Green and orange	C801K.5206	20 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 fluorescence *in situ* Hybridisation (FISH) technique in cytological specimens or FFPE tissue sections. In extensive tests and field application, the REMBRANDT® FISH kits were proven to be very robust, and FISH results were highly reproducible. For the specific intended use of a REMBRANDT® FISH detection assay, please see the product-specific datasheet.

1.2 The FISH principle

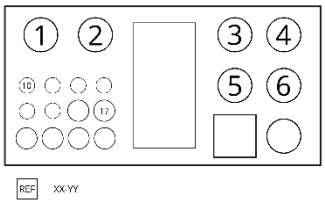
FISH enables the detection of specific DNA or RNA sequences in histological and cytological specimens, without losing the often very essential morphological details. The principle of FISH is based on a "reaction" (= hybridization) between a specifically labeled DNA or RNA sequence (= probe) and a DNA or RNA sequence present in the sample (= target). In case of matching sequences, a hybrid between the probe and target will be formed. Non-specific hybrids are washed out by the stringency wash procedure (PanWash 4). The formed hybrids can easily be visualized via fluorescent microscopy. The REMBRANDT® probes are labeled with fluorochromes; the fluorochromes used in the kits depend on specific probe(s) in the kit (Arsham et al., 2017).

1.3 Controls

1.4 Contents of a REMBRANDT® FISH detection assay

Application: cytological specimen

Contents REMBRANDT® FISH detection assays



Pos.	Contents	Product label		Vol.		REF
1	Pepsin diluent	DIGEST	PEPSIN DIL	15	ml.	R018R.0000
2	Pepsin powder	DIGEST	PEPSIN POW	1	gr.	R011R.0000
3	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
4	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
5	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
6	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
10	Product specific probe	PROBE	SPECIFIC PROBE	75 150	µl µl	XXXXX.YYYY
17	Mounting medium fluorescence	SUPPORT	MOUNT FLU	1	ml.	Z000R.0050

Application: FFPE tissue sections

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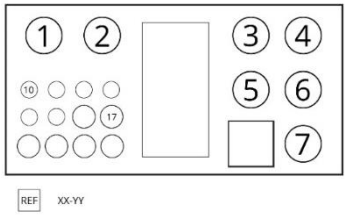
Contents REMBRANDT® FISH detection assays



Pos.	Contents	Product label		Vol.		REF
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4	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
5	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
6	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
7	Pre-treatment buffer	PRE-TREAT	Na-Citrate 100x	15	ml.	R026R.0000
10	Product specific probe	PROBE	SPECIFIC PROBE	75 150	µl µl	XXXXX.YYYY
17	Mounting medium fluorescence	SUPPORT	MOUNT FLU	1	ml.	Z000R.0050

1.5 Materials required but not included

- Xylene for dewaxing paraffin sections
- Fixative for cytological specimens
- Distilled or deionized water
- Ethanol series
  - 100% Ethanol
  - 96% Ethanol
  - 70% Ethanol
- HCl 0.01 M
- Phosphate buffered saline (PBS); pH 7.2-7.4
- Pipettes and tips to deliver 10-1000 µL
- Immersion oil for fluorescence microscopy
- Timer(s)
- Fluorescence microscope 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
- Microwave (optional)

1.6 Single product catalogue number, storage and shelf life

REMBRANDT® probe mix; 1 vial			**Catalogue No**	CXXXP.XXXX
Quantity	:	75 or 150 µl; 5 or 10 assays/kit	**Storage**	2 – 8°C
Composition	:	specific probe DNA fluorescently labeled, ready to use in hybridization buffer.		

Pepsin digestion reagent; 1 vial			**Catalogue No**	R011R.0000
Quantity	:	1 gr; powder to be dissolved in 8 mL deionized water, to be aliquoted in portions of e.g. 1200 µl or 1200 µl depending on the number of slides per test run	**Storage** powder dissolved solution	 2 – 25°C -20°C
Composition	:	Pepsin powder		

Pepsin diluent; 1 vial	**Catalogue No**	R018R.0000

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Quantity	:	15 mL 1M to be diluted 100x with deionized water to 0.01 M HCl	**Storage** 1 M HCl 0.01 M HCl	 2 – 25°C 2 – 25°C
Composition	:	HCl, 1M		

Concentrated PanWash 4, 25x SSC; 4 vials			**Catalogue No**	R025R.0000
Quantity	:	15 mL concentrated solution to be diluted 12.5x (= 2x SSC) with deionized water and confirm (and adjust if needed) to pH 7.4	**Storage** conc. solution diluted solution	 2 – 25°C ambient temp.
Composition	:	Sodium chloride, sodium citrate and surfactant		

Concentrated Pre-treatment buffer, 100x Na-Citrate; 1 vial (optional)			**Catalogue No**	R025R.0000
Quantity	:	15 mL concentrated solution to be diluted 100x (= 1x Na-Citrate) with deionized water and confirm (and adjust if needed) pH 6.0	**Storage** conc. solution diluted solution	 2 – 25°C ambient temp.
Composition	:	Natrium citrate		

Mounting medium; 1 vial			**Catalogue No**	Z000R.0050
Quantity	:	2 mL	**Storage**	2 – 8°C
Composition	:	DAPI (4,6-diamidino-2-phenylindole), DABCO (diazabicyclo-2.2.2- octane) in TRIS buffer and glycerol		

– Store kit and its contents at 2-8°C.
– Store the dissolved and aliquoted reagents at recommended temperatures (see above).
– When used and stored as indicated, the kit is stable until the expiry date printed on the box.

1.7 Safety precautions

– The hybridization buffer in which the probes are mixed contains formamide, which is considered as a teratogen; contact with skin and mucous membranes should be avoided.
– The Mounting medium contains DAPI (4,6-diamino-2-phenylindole) and 1,4-phenylenediamine which is a possible respiratory and dermal sensitizer; contact with skin and mucous membranes should be avoided.
– 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.

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- 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.
- 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.
- Homogenise all solutions before use.
- Homogenise 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 4 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 Hybridisation 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.
- Fluorochromes are light sensitive; therefore, all steps that do not require manipulation should be performed under exclusion of light.

1.9 Preparation of supplied reagents in advance

Pepsin stock solution:
Dissolve the pepsin digestion reagent in 8 mL distilled or deionized 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 number of slides that are subject to the 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 deionized 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

PanWash 4:
- Dilute a vial of the supplied 15 mL PanWash 4, 25x SSC to **2x SSC** with 172.5 mL deionized or distilled water to a total of 187.5 ml (concentration is 2x SSC).
- Dilute 0.8 mL of a vial of the supplied PanWash 4, 25x SSC to **0.1x** SSC with 199.2 mL deionized or distilled water to a total of 200 ml (concentration is 0.1x SSC).
Before use:
a) Fill staining jars with SSC solution.
b) Pre-heat 2x SSC at 42°C and 0.1x SSC at 61°C (if applicable)
Do not re-use

Pre-treatment buffer (included depending on assay type; see product-specific datasheet):
Dilute the supplied 15 mL Pre-treatment buffer, 100x Na-Citrate to 1x Na-Citrate with 1485 mL deionized or 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%)

PBS (phosphate buffered saline)/ Tween® 20 (0.05%)
- Deionized water:
  950 ml
- Sodium chloride (NaCL):
  8 g
-Potassium chloride (KCl):
  0.2 g

- di-Sodium hydrogen phosphate di-hydrate ($Na_2HPO_4.2H_2O$):
  1.78 g

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- Potassium di-hydrogen phosphate (KH_2PO_4):
  0.24 g
-Tween® 20
  0.5 ml
Adjust pH to 7.4 +/- 0.2 and adjust to 1000 mL with deionized water
Do not re-use

1.11 Microscope and accessories

– **Microscope**

A fluorescence microscope is needed to validate the *in situ* hybridization results.

– **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 the 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.

– **Filters**

Most, if not all microscope manufacturers, can deliver the needed single and multi-band pass filters. The single and multi-band pass filters needed for the REMBRANDT® FISH detection assays are:

Filter set for DAPI	
excitation	G 365 nm
beam splitter	FT 395 nm
emission	LP 420 nm

Filter set for green fluorochrome	
Excitation	BP 450 nm – 490 nm
beam splitter	FT 510 nm
emission	BP 515 nm – 565 nm

Filter set for orange fluorochrome	
excitation	BP 546 nm /12
beam splitter	FT 580
emission	LP 590 nm

Triple band filter set (DAPI / FITC / TRITC)

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– **Immersion oil**

The use of quality immersion oil is recommended; the immersion oil should have low auto-fluorescence and be suited for use in fluorescence microscopy.

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Chapter 2 REMBRANDT® FISH Protocol

- All incubation steps should be performed in a closed (dark) 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 commonly used for cytological specimens, it preserves the morphology and target DNA. Other fixation methods such as Carnoy's fixative are also applicable.

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 FISH, 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 deionized water and continue with pre-treatment.

2.2 Pre-treatment of specimen

Cytological specimen
- Incubate slides in pre-heated proteolytic work solution at 37 °C (i.e. 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.

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Paraffin-embedded tissue sections

Pre-treatment procedures are necessary to make the DNA accessible for the probes and to obtain reliable and reproducible results.

- Place the slides in a jar filled with pre-treatment buffer (R026R.0000), place the jar in a microwave set at i.e. 900W and incubate up until boiling; subsequently, reset the microwave at 180W and proceed with the incubation for 10 minutes followed by a 20-minute cooldown period at room temperature; all in the same solution. Flush wash slides in deionized water at room temperature.
- Incubate slides in pre-heated proteolytic work solution at 37 °C (1.25 mg/ml) for 15 minutes.
- Flush wash in deionized water, followed by dehydration in graded ethanol series (ethanol 70%, 96%, 96%, 100% and 100%), 1 minute each and air-dry slides for 15 minutes.

Do not treat more than 5 slides at the same time, because the temperature of the pre-heated solutions may drop dramatically, thus causing incomplete pre-treatment. Additionally, allow the slides to air-dry as recommended; otherwise sections will be lost.

2.3 Hybridisation procedure and post-hybridization washes

Denaturation and Hybridisation

- Homogenize probe solution and spin briefly. Apply 10-15 µl of probe solution to each specimen. Cover all specimens with a cover slip (avoid air bubbles).
- Place slides on an 80 °C hotplate or other heating device and incubate for 3 minutes in case of cytological specimen, and 10 minutes in case of FFPE tissue sections (denaturation).

Work in a pre-set order to ensure that all slides have been incubated at 80 °C for the exact same time! Do not denature more than 5 slides at the same time, because the temperature of the heating device may drop dramatically, thus causing incomplete denaturation.

- Transfer slides into a moist environment and incubate in the dark for 16 hours at 37 °C.

Differentiation (stringent wash) and rinsing

- Remove coverslips by submerging the slides in PBS at room temperature. Soak the slides until the coverslips fall off.
- Incubate slides in pre-heated PanWash 4, **buffer concentration and incubation temperature differ per specific product** (see product-specific datasheet).
- Incubate slides in PBS at room temperature for 1 minute.
- Dehydrate in graded ethanol series. Air-dry slides for 15 minutes (in the dark).

Do not incubate more than 5 slides at the same time in PanWash 4 (2x SSC and 0.1x SSC), because the temperature of PanWash 4 may drop dramatically, causing wrong stringency conditions.

Coverslipping

Mount slides by applying 1-2 drops of the supplied mounting medium (Z000R.0050) and coverslip.

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Chapter 3 Interpretation of Results

3.1 Guidelines for interpretation

- For specific interpretation guidelines and scoring criteria, see REMBRANDT® FISH detection product-specific datasheet.
- For assessment, it is important to select representative areas of the specimen.
- Over-digestion can lead to DNA loss and affect the gene signal numbers in individual nuclei. Score only nuclei that have relatively intact nuclear borders.

3.2 Quality control

Positive control

It is advised to run a positive and negative control simultaneously with each FISH assay. As a positive control, one may also use validated in-house specimens. If the controls fail to demonstrate the expected staining, the result on the test specimen must be validated as invalid.

Assay requirements

- Noise-to-signal percentage
  A FISH probe is detected via fluorescent microscopy. The probe will emit a fluorescent signal. However, a fluorescent signal can also be visualized in the cells. Therefore, the noise-to-signal cut-off needs to be determined for FISH probes. If the fluorescent signal in the background is too close to the actual probe signal, the probe is not useable in diagnostics. For good signal interpretation, the noise-to-signal percentage should be ≤40%.
- Hybridization efficiency
  Signals may only be interpreted if the probe signals are visualized in ≥98% of the cells.

For additional requirements, see the product-specific datasheet.

Invalid results

- In case the control does not stain adequately, the results of the test slides cannot be accepted. The run should be repeated to rule out procedural errors. Please check the troubleshooting section below for further possibilities.
- Poor cell morphology may lead to unacceptable noise-to-signal percentage. In these cases, we advise assessing 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 nuclear borders are lost or persistent green or red auto-fluorescence masks true signals, the enzymatic digestion was not optimal and the test 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® FISH 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.
- Medical decisions may not be taken based on this test. This is a research-use-only product.
- Sample fixation methods should be performed according to the recommended sample preparation methods.
- Many factors can influence the performance of the FISH 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® FISH detection assays was tested and validated using the exact procedure as listed in the product-specific data sheets; modifications to the procedure may alter the performance characteristics.
- In case the sampling, sampling method, quality, sample preparation, reagents used, controls and procedure followed are not optimal, the REMBRANDT® FISH detection assay test results are not to be relied on.
- The medical profession should be aware of risks and factors influencing the intensity, the absence, or the presence of FISH 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, and professional and be able to interpret the test results.
- The specific FISH detection assay should only be used for the loci the probe targets.

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Product in combination with other devices

The REMBRANDT® FISH 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.

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® FISH detection assays (Duffy et al., 2012) (Arsham et al., 2017).

It is of course always possible that you encounter a problem that 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 the correct concentration of pre-treatment solutions is used → Make sure pre-treatments are performed at the correct temperatures → Make sure dehydration steps are performed as recommended → Make sure that all pre-treatment steps are performed
	■ Hybridization procedure	→ Homogenize the probe solution before applying probe on the section
	■ PanWash 4 temperature and incubation time	→ Make sure correct temperatures are maintained and that the incubation time of the washing step is followed
	■ Coverslips removed with force	→ Make sure the 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 visualize if any
	■ Insufficient dehydration	→ Prepare fresh dehydration solutions
	■ Photo-damage to the fluorescently labled probe	→ Try to prevent prolonged light exposure and work in a dark environment if applicable
	■ No target sequence present	→ Use appropriate controls

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	■ Microscope and accessories	→ Check filter set → Check lamps → Check the running hours of the lamp → Check lamp alignment → Check and clean lenses and mirror → Call microscope technical service → 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 the temperature is 80 °C
	■ Denaturation step too long	→ Denature no longer than 5 minutes
	■ Washing temperature	→ Make sure correct temperatures are maintained → 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
	■ The tissue section is too thin	→ The optimal thickness of the tissue is 2-4 µm
	■ Wrong (side of) glass slide used	→ Use only plus coated glass slides
	■ Pepsin concentration is too high	→ Make sure the correct concentration of pepsin is used
	■ A proteolytic pre-treatment step too long	→ Reduce time of proteolytic pre-treatment step
	■ Denaturation	→ Make sure the temperature is 80 °C → Denature no longer than 10 minutes

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	■ Coverslips removed with force	→ Make sure the 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 fixative3 and check fixation time (prevent over-fixation)
	■ Deparaffinisation	→ Renew dewax series
	■ Pre-treatment	→ Make sure the correct concentration of pre-treatment solutions is used → Make sure pre-treatments are performed at the correct temperatures → Make sure dehydration steps are performed as recommended → Make sure that all pre-treatment steps are performed
	■ Denaturation	→ Make sure the temperature is 80 °C
	■ Hybridization procedure	→ Homogenize the probe solution before applying probe on the section
	■ PanWash 4 temperature and incubation time	→ Make sure correct temperatures are maintained and that the incubation time of the washing step is followed
	■ Air bubbles during denaturation and hybridization	→ Make sure that when covering the section with a cover slip, air bubbles are not present
	■ Insufficient amount of probes used	→ Make sure that a sufficient amount of probe is used to cover the section
	■ No target sequence present	→ Use appropriate controls
	■ Photo damage to the fluorescently labeled probe	→ Try to prevent prolonged light exposure and work in a dark environment if applicable
	■ Microscope and accessories	→ Check filter sets → Check lamps → Check running hours of lamp → Check lamp alignment → Check and clean lenses and mirror → Call microscope technical service

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Non-specific background staining and signal variation	■ Tissue section is too thick	→ The optimal thickness of the tissue is 2-4 µm
	■ Tissue crumbled	→ Make sure the tissue is stretched completely
	■ Deparaffinisation	→ Dewax series
	■ Denaturation temperature too high	→ Make sure the temperature is 80 °C
	■ Hybridization: uneven distribution of probe due to air bubbles/uneven section	→ Repeat the procedure on a new section
	■ Denaturation step too long	→ Denature no longer that 10 minutes
	■ Drying out of the section	→ Incubate all procedure steps in a moisturized environment; prevent evaporation
	■ Washing temperature	→ Make sure correct temperatures are maintained → 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.

Immaterial property information

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REMBRANDT®

In situ Hybridisation and Detection

ISH detection assays-*v5.2023*

ISH kit		
Label	**Product number**	**# assays**
Biotin	CxxxK.0100.05	5 T
Biotin	CxxxK.0100.10	10 T
Digoxigenin	CxxxK.9900.05	5 T
Digoxigenin	CxxxK.9900.10	10 T
Biotin and Digoxigenin	CxxxK.0199.05	5 T
Biotin and Digoxigenin	CxxxK.0199.10	10 T

RUO

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

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 conjugated 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-specific hybrids are washed out by the stringency wash procedure (PanWash 4). The formed hybrids can easily be visualized after detection with corresponding antibodies by fluorescent or brightfield microscopy. The REMBRANDT® 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).

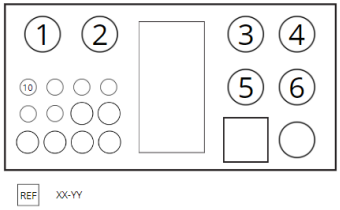
1.3 Controls

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® ISH detection assay

Application: cytological specimen

Contents REMBRANDT® ISH detection assays



Pos.	Contents	Product label		Vol.		REF
1	Pepsin diluent	DIGEST	PEPSIN DIL	15	ml.	R018R.0000
2	Pepsin powder	DIGEST	PEPSIN POW	1	gr.	R011R.0000
3	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
4	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
5	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
6	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
10	Product specific probe	PROBE	SPECIFIC PROBE	75 150	µl µl	XXXXX.YYYY

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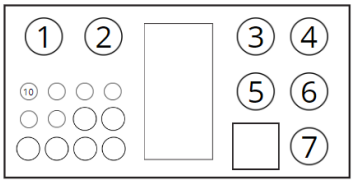
Application: FFPE tissue sections

Contents REMBRANDT® ISH detection assays



REF XX-YY

Pos.	Contents	Product label		Vol.		REF
1	Pepsin diluent	DIGEST	PEPSIN DIL	15	ml.	R018R.0000
2	Pepsin powder	DIGEST	PEPSIN POW	1	gr.	R011R.0000
3	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
4	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
5	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
6	PanWash 4	PAN-WASH 4	SSC 25X	15	ml.	R025R.0000
7	Pre-treatment buffer	PRE-TREAT	Na-Citrate 100x	15	ml.	R026R.0000
10	Product specific probe	PROBE	SPECIFIC PROBE	75	µl	XXXXX.YYYY
				150	µl	

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
- HCl 0.01 M
- Phosphate buffered saline (PBS); pH 7.2-7.4
- Pipettes and tips to deliver 10-1000 µL
- Immersion oil for fluorescence or brightfield microscopy
- Timer(s)
- Fluorescence microscope 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
- Microwave (optional)

1.6 Single product catalogue number, storage and shelf life

REMBRANDT® probe mix; 1 vial		**Catalogue No**	CXXXP.XXXX
Quantity	: 75 or 150 µl; 5 or 10 assays/kit	**Storage**	2 – 8°C
Composition	: specific conjugated probe DNA, ready to use in hybridisation buffer.		

Pepsin digestion reagent; 1 vial		**Catalogue No**	R011R.0000
Quantity	: 1 gr; powder to be dissolved in 8 mL deionised water, to be aliquoted in portions of e.g. 1200 µl or 1200 µl depending on the amount of slides per test run	**Storage** powder dissolved solution	 2 – 25°C -20°C
Composition	: Pepsin powder		

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Pepsin diluent; 1 vial			**Catalogue No**	R018R.0000
Quantity	:	15 mL 1M to be diluted 100x with deionised water to 0.01 M HCl	**Storage** 1 M HCl 0.01 M HCl	 2 – 25°C 2 – 25°C
Composition	:	HCl, 1M		

Concentrated PanWash 4, 25x SSC; 4 vials			**Catalogue No**	R025R.0000
Quantity	:	15 mL concentrated solution to be diluted 12.5x (= 2x SSC) with deionised water and confirm (and adjust if needed) to pH 7.4	**Storage** conc. solution diluted solution	 2 – 25°C ambient temp.
Composition	:	Sodium chloride, sodium citrate and surfactant		

Concentrated Pre-treatment buffer, 100x Na-Citrate; 1 vial (optional)			**Catalogue No**	R025R.0000
Quantity	:	15 mL concentrated solution to be diluted 100x (= 1x Na-Citrate) with deionised water and confirm (and adjust if needed) pH 6.0	**Storage** conc. solution diluted solution	 2 – 25°C ambient temp.
Composition	:	Natrium citrate		

- Store kit and its contents at 2-8°C.
- Store the dissolved and aliquoted reagents at recommended temperatures (see above).
- When used and stored as indicated, the kit is stable until the expiry date printed on the box.

1.7 Safety precautions

- The hybridisation buffer in which the probes are mixed contains formamide, which is considered as a teratogen; contact with skin and mucous membranes should be avoided.
- 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 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.
- 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.

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- Homogenise all solutions before use.
- Homogenise 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 4 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 Hybridisation 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

PanWash 4:
- Recommended for non-stringent washes:

Dilute a vial of the supplied 15 mL PanWash 4, 25x SSC to **2x SSC** with 172.5 mL deionised or distilled water to a total of 187.5 ml (concentration is 2x SSC).
- Recommended for stringent washes:

Dilute 0.8 mL of a vial of the supplied PanWash 4, 25x SSC to **0.1x** SSC with 199.2 mL deionised or distilled water to a total of 200 ml (concentration is 0.1x SSC).
Before use:

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a) Fill staining jars with SSC solution and pre-heat to the desired temperature.
Do not re-use

Pre-treatment buffer (included depending assay type; see product specific datasheet):
Dilute the supplied 15 mL Pre-treatment buffer, 100x Na-Citrate to 1x Na-Citrate with 1485 mL deionised or 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%)

PBS (phosphate buffered saline)/Tween® 20 (0.05%)
- Deionised water:
  950 ml
- Sodium chloride (NaCL):
  8 g
-Potassium chloride (KCl):
  0.2 g
- di-Sodium hydrogen phosphate di-hydrate ($Na_2HPO_4.2H_2O$):
  1.78 g
- Potassium di-hydrogen phosphate (KH_2PO_4):
  0.24 g
-Tween® 20
  0.5 ml

Adjust pH to 7.4 +/- 0.2 and adjust to 1000 mL with deionised water
Do not re-use

1.11 Microscope and accessories

- **Microscope**
  A fluorescence or brightfield microscope is needed to validate the *in situ* hybridisation 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**

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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® ISH Protocol

- <u>In case of detection with fluorescent antibodies:</u> All incubation steps should be performed in a closed (dark) incubation chamber/staining jar 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 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.

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 pre-treatment.

2.2 Pre-treatment of specimen

Cytological specimen
- 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.

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Paraffin embedded tissue sections

For making the DNA accessible for the probes and to obtain reliable and reproducible results, pre-treatment procedures are necessary.

- Place slides in jar filled with pre-treatment buffer (R026R.0000), place the jar in a microwave set at i.e. 900W and incubate up until boiling, subsequently reset microwave at 180W and proceed with the incubation for 10 minutes followed by a 20 minute cool down period at room temperature; all in the same solution. Flush wash slides in deionised water at room temperature.
- Incubate slides in pre-heated proteolytic work solution at 37 °C (1.25 mg/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.

Do not treat more than 5 slides at the same time, because the temperature of the pre-heated solutions may drop dramatically, thus causing incomplete pre-treatment. Additionally, allow the slides to air-dry as recommended; otherwise sections will be lost.

2.3 Hybridisation procedure and post-hybridization washes

Denaturation and Hybridisation

- Homogenize probe solution and spin briefly. Apply 10-15 µl of probe solution to each specimen. Cover all specimens with a cover slip (avoid air bubbles).
- Place slides on an 80 °C hotplate or other heating device and incubate for 3 minutes in case of cytological specimen and 10 minutes in case of FFPE tissue sections (denaturation).

Work in a pre-set order to ensure that all slides have been incubated at 80 °C for the exact same time! Do not denature more than 5 slides at the same time, because the temperature of the heating device may drop dramatically, thus causing incomplete denaturation.

- Transfer slides into a moist environment and incubate for 16 hours at 37 °C.

Differentiation (stringent wash) and rinsing

- Remove coverslips by submerging the slides in PBS at room temperature. Soak the slides until the coverslips fall off.
- Incubate slides in pre-heated PanWash 4.

The concentration of PanWash 4 and the temperature may strongly depend on the detection system used. Recommend washes for non-stringent conditions are: 2x 5 minutes in 2x SSC at 42˚C. For a stringent wash: 2x 5 minutes in 2x SSC at 42˚C followed by 2x 5 minutes in 0.1x SSC at 61˚C. Do not incubate more than 5 slides at the same time in PanWash 4 (2x SSC and 0.1x SSC), because the temperature of PanWash 4, may drop dramatically, causing wrong stringency conditions.

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- Incubate slides in PBS at room temperature for 1 minute.
- Dehydrate in graded ethanol series. Air-dry slides for 15 minutes (in the dark).

Recommended antibody detection systems

The appropriate detection system should be evaluated by the end-user. Recommended detection systems are listed below:

Digoxigenin detection	Biotin detection
R003R.0000 REMBRANDT® Sheep aDig-AP conjugate	R041R.0000 REMBRANDT® Goat aBio-AP Fab conjugate
R004R.0000 REMBRANDT® Sheep aDig-HRP conjugate	R042R.0000 REMBRANDT® Goat aBio-HRP Fab conjugate

After incubation with conjugated antibodies, use an appropriate detection system. Recommended chromogenic detection systems are listed below:

AP detection	HRP detection
R008R.0000 REMBRANDT® NBT/BCIP substrate	R007R.0000 REMBRANDT® AEC substrate + R010R.0000 REMBRANDT® AEC buffer

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Chapter 3 Interpretation of results

3.1 Guidelines for interpretation

- For specific interpretation guidelines and scoring criteria, see REMBRANDT® ISH detection product specific datasheet.
- For assessment it is important to select representative areas of the specimen.
- Over digestion can lead to DNA loss and affect the gene signal numbers in individual nuclei. Score only nuclei that have relatively intact nuclear borders.

3.2 Quality control

Positive control

It is advised to run a positive and negative control simultaneously with each ISH assay. As a positive control, one may also use validated in-house specimen. If the controls fail to demonstrate the expected staining, the result on the test specimen must be validated as invalid.

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 hybridisation 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® ISH 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.
- Medical decisions may not be taken based on this test. This is a research use only product.
- Sample fixation methods should be performed according to the recommended sample preparation methods.
- 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 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® ISH 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 ISH detection assay should only be used for the loci the probe targets.

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Product in combination with other devices

The REMBRANDT® ISH 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® ISH 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 → Make sure pre-treatments are performed at correct temperatures → Make sure dehydration steps are performed as recommended → Make sure that all pre-treatment steps are performed
	■ Hybridisation procedure	→ Homogenize probe solution prior to applying probe on the section
	■ PanWash 4 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

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	■ Microscope and accessories	→ Check filter sets → Check lamps → Check running hours of lamp → Check lamp alignment → Check and clean lenses and mirror → Call microscope technical service → 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 → 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 → Denature no longer than 10 minutes

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	■ 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 → Make sure pre-treatments are performed at correct temperatures → Make sure dehydration steps are performed as recommended → Make sure that all pre-treatment steps are performed
	■ Denaturation	→ Make sure temperature is 80 °C
	■ Hybridisation procedure	→ Homogenize probe solution prior to applying probe on the section
	■ PanWash 4 temperature and incubation time	→ Make sure correct temperatures are maintained and that incubation time of washing step is followed
	■ Air bubbles during denaturation and hybridisation	→ 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 → Check lamps → Check running hours of lamp → Check lamp alignment → Check and clean lenses and mirror → Call microscope technical service

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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
	■ Hybridisation: 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 → 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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