Quick Reference Guide

Upon receipt of the kit dissolve pepsin powder (black) in 4 mL distilled/deionized water, aliquot in 150 µL batches and freeze at -20°C.

PRETREATMENT OF PARAFFIN SECTIONS	***INCUBATION TIME***
1. Cut 4-6 µm sections and collect on treated glass slide	
2. Heat slides	2 - 16 hours at 56-60°C
3. Dewax in fresh xylene	2 x 10 min.
4. Soak slides in 100% ethanol and air dry	5 min.

PROTEOLYTIC TREATMENT	
1. Dilute the 1N HCl pepsin diluent (transparent)	
2. Dilute thawed proteolytic stock solution in diluted HCl and incubate each specimen with 300-400 µL:	
paraffin: 100x in 0.1N HCl; add 50 µL to 5 mL 0.1N HCl	30 min. on a 37°C heating block
cytological:25,000x in 0.01N HCl;add 4 µL to 100 mL 0.01N HCl	10 min on a 37°C heating block
frozen: 50,000x in 0.01N HCl; add 2 µL to 100 mL 0.01N HCl	10 min. on a 37°C heating block
3. Discard excess proteolytic work solution	
4. Dehydrate slides in graded ethanol and air dry	3 x 1 min.

HYBRIDIZATION PROCEDURE	
1. Apply 1 drop or 20 µl of probe solution per specimen. Cover with coverslip.	
2. Denature	5 min. at 95°C hotplate
3. Hybridize	2 hours at 37°C incubator
4. Remove coverslips by soaking slides in TBS buffer	10 min.
5. Apply 5-6 drops of PanWash (white) to each specimen except the positive control	15 min. on a 37°C heating block
6. Wash all slides in TBS buffer	3 x 1 min.

DETECTION AND STAINING PROCEDURE	
1. Apply 2-3 drops of the conjugate (red) to each specimen	30 min. on a 37°C heating block
2. Soak slides in TBS buffer	3 x 1 min.
3. Soak slides in deionized water	1 min.
4. Apply 2-3 drops of NBT/BCIP substrate (blue) to each specimen and incubate in dark	5-15 min. on a 37°C heating block
5. Tap off excess substrate solution and wash slides in distilled or deionized water	3 x 1 min.
6. Optional: apply 2-3 drops of counterstain (orange) to each specimen	1 min.
7. Wash slides in deionized water	3 x 1 min.
8. Mount sections for microscopic evaluation	

PanPath

REMBRANDT®

In Situ Hybridization and Detection

DISH & AP Detection Kit

DISH-AP kit for the detection of	**Biotin Label Cat.no.**	**Digoxigenin label Cat.no.**	**# Assays**
HPV screening	HKB18000	HKD38000	40
HPV typing	HKB18003	HKD38003	40
Cytomegalovirus	HKB18047	HKD38047	40
Epstein-Barr virus	HKB18049	HKD38049	40
Herpes simplex virus	HKB18056	HKD38056	40

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Patents pending; see inside back cover.

Intended use

REMBRANDT® has been designed for the processing of paraffin embedded tissue sections, cytological specimens and frozen (cryostat) sections, to detect a specific sequence of DNA or RNA by using the *In Situ* Hybridization (ISH) technique. Unless explicitly stated otherwise, all products are for research purposes only.

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" (= hybridization) between a specifically labeled DNA sequence (= probe) and a DNA or RNA sequence present in the sample (= target). In case of matching sequences, a hybrid will be formed which can easily be visualized by a specific staining procedure, i.e. substrate conversion by enzyme-conjugated antibodies. This conversion, like the combination of NBT/BCIP and Alkaline Phosphatase (AP) conjugated αDIG/αBIO-Fab fragments provided with this kit, will yield a detectable and colored precipitation. The ISH technique is highly sensitive, specific, fast and easy to perform. Moreover, no radioactivity is involved. Therefore, REMBRANDT® is the ultimate user-friendly tool for performing ISH.

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, and positive control slides containing the desired target DNA. In addition, it is recommended to include sections from the specimen under investigation for use with the provided positive and negatives control probes. Additional control slides and probes are available from PanPath; please contact your local supplier.

Contents of a REMBRANDT® DISH & AP Detection Kit

Black vial	:	Pepsin digestion reagent	1 gram
Transparent vial	:	Pepsin diluent (1N HCl solution)	15 mL
Yellow/Purple vial	:	Specific* BIO or DIG labeled DNA probe(s)	0.8 mL
Pink vial	:	DISH positive control oligo probe	0.8 mL
Green vial	:	DISH negative control DNA probe	0.8 mL
White vial	:	PanWash (Differentation reagent)	2x15 mL
Red vial	:	AP-conjugated αDIG or αBIO	15 mL
Blue vial	:	NBT/BCIP substrate	15 mL
Orange vial	:	Nuclear Fast Red counterstain	15 mL
Pouches	:	TBS buffer salt	2 pcs

Immaterial property information

The probes in this product are labeled with the Universal Linkage System (ULS®). The Universal Linkage System (ULS®) technology is covered by an international patent family for the linkage of any label to bio-organic molecules, owned by KREATECH Biotechnology BV, The Netherlands. This product or the use of this product may be covered by one or more patents of KREATECH Biotechnology BV, including, but not restricted to, the following: EP 0539466; US 5,580,990; US 5,714,327; WO 92/01699; WO 96/35696; WO 98/15564.

Digoxigenin (DIG) labeling and detection is protected by international patents of Roche Molecular Biochemicals. This product is sold under a license of Roche Molecular Biochemicals. This product or the use of this product may be covered by one or more patents of Roche Molecular Biochemicals, including the following: EP 0324474, US 5,354,657.

REMBRANDT® is a registered tradename of KREATECH Biotechnology BV., Amsterdam, The Netherlands.

II.5 Non-specific background staining

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

Possible causes	Remedies
■ Tissue section too thick.	→ Optimal thickness of the tissue is 4-6 µm.
■ Tissue crumbled.	→ Make sure tissue is stretched completely.
■ Deparaffinization.	→ Dewax series
■ Denaturation temperature too high.	→ Make sure temperature is 95 ± 5°C.
■ Denaturation step too long.	→ Denature no longer than 5 minutes.
■ Drying out of the section.	→ Hybridize in a moisturous environment.
■ Washing temperature.	→ Make sure temperature is 37 ± 2°C.
■ Substrate incubation step too long.	→ Shorten incubation time with 5 minutes.
■ Endogenous peroxidase.	→ Inactivate endogenous peroxidase by incubating tissue sections in 3% H_2O_2/H_2O for 15 minutes at room temperature prior to the digestion step.
■ Endogenous alkaline phospatase.	→ Inactivate endogenous alkaline phospatase by incubating sections in substrate solution to which 4 mg of levamisol is added.

II.6 Cross Hybridization

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

Box A	:	Coated glass slides	50 pcs
Box B	:	Coverslips	100 pcs
Box C**	:	Positive control slides	2 pcs

* For specific probe specifications see page 9.
** REMBRANDT kits for HSV (cat.no. HKB18056 and HKD38056) contain 2 boxes: Box C and D containing 1 pcs of a HSV positive control slide each

Materials required but not included

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

Storage and shelf life

– Store all reagents at 2-6°C upon receipt of the kit.
– Store the dissolved and aliquoted pepsin reagent at -20°C, stable for at least 1 year when kept frozen.
– Store the dissolved TBS buffer at 2-6°C when not in use.
– When used and stored as indicated, the kit is stable until the expiration date printed on the box.

Safety precautions

– Some reagents contain Na-azide or thimerosal (preservation) which can cause irritation when exposed to skin or mucous membranes. The concentrations of these preservations, however, are very low (< 0.1%). If reagents come into contact with skin or eyes, wash with large volumes of clean water.
– Never pipet solutions by mouth.
– The control slide in the kit contains pathogenic material fixed with 4% paraformaldehyde making specimens noninfectious: however, we advise taking normal precautions for handling infectious organisms.

Performance precautions

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

Preparation of reagents in advance

Pepsin digestion reagent: dissolve this proteolytic reagent (black) in 4 mL of distilled or deionized water (upon receipt of the kit). Aliquot in e.g. portions of approximately 50 µL and freeze at -20°C.

Pepsin diluent:
For paraffin sections: dilute the 1N HCl solution (transparent) 10x with distilled or deionized water into a 0.1N HCl solution. For cytological specimens and frozen sections: dilute the 1N HCl solution 100x into a 0.01N HCl solution.

TBS buffer salt: dissolve 1 pouch in 1000 mL distilled or deionized water. Dissolve the salt completely and keep the buffer free from contamination.

Preparation of the proteolytic work solution

Prepare between 300 and 400 µL per section of 1 cm^2. Make fresh work solution just before use and discard non-used solution!

Paraffin sections: dilute aliquoted proteolytic reagent 100x in 0.1N HCl, e.g. add 50 µL to 5 mL 0.1N HCl and mix.

Cytological specimens: dilute aliquoted proteolytic reagent 25,000x in 0.01N HCl, e.g. add 4 µL to 100 mL 0.01N HCl and mix.

Frozen sections: dilute aliquoted proteolytic reagent 50,000x in 0.01N HCl, e.g. add 2 µL to 100 mL 0.01N HCl and mix.

II.3 Negative staining of the positive control

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

II.4 Positive staining of the negative control

Possible causes	Remedies
■ Drying out of the section.	→ Hybridize in a moisturous environment.
■ Washing procedure.	→ Make sure temperature is 37 ± 2°C. → Depending on GC%, make sure correct PanWash (Differentiation reagent) is used.
■ Contamination with positive control probe or specific probe.	→ Make sure that the positive control probe is the latest to be applied to the section.

II.2 Weak or no staining on a suspected positive sample

Possible causes	Remedies
■ Tissue fixation.	→ Only use buffered formalin fixative.
■ Deparaffinization.	→ Refresh dewax series.
■ Digestion.	→ Make sure correct concentration of pepsin is used. → Make sure digestion takes place at 37°C.
■ Denaturation.	→ Make sure temperature is 95 ± 5°C.
■ Interfering internal structures of probes.	→ In case of RISH procedures, warm up probe solution at 85°C for 5 min. before usage.
■ Hybridization procedure.	→ Homogenize probe solution prior to applying probe on the section.
■ Washing temperature.	→ Make sure temperature is 37 ± 2°C.
■ Detection procedure.	→ Make sure temperature is 37 ± 2°C. → Make sure to incubate in the dark.
■ Low amount of target DNA.	→ Overnight hybridization.
■ Color precipitate washed away	→ Make sure that proper wash and mounting media are used.

REMBRANDT® DISH & AP Detection Protocol

Specimen collection and pre-treatment

Paraffin embedded tissue sections

A standard procedure for tissue fixation and embedding usually involves the use of formalin and paraffin. The optimal tissue block size is 0.5 cm^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 a temperature of 65°C.

Sample preparation: stretch 4-6 µm paraffin sections on distilled water of 55°C without any additives and collect sections on organosilane coated glass slides. Bake the slides at 56°C - 60°C in a dry air oven for 2-16 hours. Slides can be used immediately or they can be stored at room temperature for up to 3 months. Prior to ISH, slides need to be dewaxed in fresh xylene for 2 x 10 minutes. Incomplete removal of formalin and/or paraffin may affect the result of the procedure. Place the slides in 100% ethanol for 5 minutes. Air dry the slides for approximately 5-10 minutes and start with proteolytic treatment.

Cytological specimens

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

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

Frozen sections

In general, small pieces of tissue (max. 1 cm^3) are snapfrozen 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 (6 µm) on the treated glass slides and air dry for 30 minutes. Fix the sections with a cross-linking fixative (e.g. 4% paraformaldehyde) for 10 minutes at room temperature. Dehydrate in graded ethanol, air dry and start with proteolytic treatment.

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 proteolytic work solution to each specimen. Incubate at 37°C:

paraffin sections for 30 minutes, cytological and frozen specimens for 10 minutes. Tap off proteolytic work solution and dehydrate the slides in graded ethanol series (70%, 95% and 100%). Duration of each soak is 1 minute. Air dry the slides and start with the hybridization procedure.

Hybridization procedure

Denaturation and Hybridization
Homogenize probe solution. Apply 1 drop or 20 µl of probe solution (yellow/purple) to each specimen. Apply 1 drop or 20 µl of the negative control probe (green) to each negative control specimen and apply 1 drop or 20 µl of the positive control probe (pink) to each positive control specimen. Cover all specimens with a coverslip (avoid air bubbles!). Place slides on a 95°C hotplate and incubate for 5 minutes (denaturation). Work in a preset order to ensure that slides have been incubated at 95°C for the exact same time! Transfer slides into a moist environment and incubate for 2 hours at 37°C (during the hybridization the minimum temperature should be room temperature and the maximum temperature should be 37°C).

Differentiation and washing
- Remove coverslips by submerging the slides in TBS buffer. Soak the slides until the coverslips fall off. Wash the slides in TBS buffer for 10 minutes. Take the slides out, wipe off excess buffer and dry the edges using a lint-free cloth. Please mind **NOT** to perform the differentiation step on specimens incubated with the positive control oligo probe (pink)!
- Transfer the slides onto a 37°C heating block or slide warmer. Apply 5-6 drops of PanWash (white) to each specimen, except to the positive control and 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.

Detection and staining procedure

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

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. Transfer the slides onto a 37°C heating block or slide warmer and apply 2-3 drops of NBT/BCIP substrate (blue) to each specimen. Incubate in the dark for 5-15 minutes at 37°C (examine the color development every 5 minutes with a light microscope). Remove the slides, one at a time, from the heating block. Tap off excess substrate solution. Wash the slides for 3x 1 minute in changes of distilled or deionized water. The slides are now ready to be mounted or counterstained.

II.1 No section or cells left on the slides

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

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Trouble Shooting Guide

I. Introduction

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

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

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

Counterstain procedure

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

Examining the processed slides

First, check the negative and positive controls that have been incubated with the test slides simultaneously:
- The negative control should be really negative, i.e. not show any localized color 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 color precipitations in conformity with the localization of the target DNA in the nucleus. The color should be the proper shade and must be clearly visible in the preferential cell type or tissue location.

In the test slides, start under low power magnification and focus on localization and color to see whether:
- The positivity (color precipitation) observed is localized in the cell type preferred by the virus type.
- The color is the right shade (no endogenous or formalin pigment).

Use high power magnification to see whether:
- The positive staining texture (granular, etc) and demarcation are conforming the positive control.

Limitations of the procedure

Failure in detection can be due to improper sampling, handling, fixation or processing, or presence below the sensitivity of this assay. Negative results therefore do not rule out any possibility of a positive infection.

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Probe specifications

Cat. No.	Type	Assays	Probe specification	Contents
HKB18000 HKD38000	HPV screening	40	Pan HPV DNA probe: size: 100-300 bp, vector: mix of pBR322 and pSP, region: mix of total genomes (7-8 kb) and DNA containing the conserved HPV region	1 x 0.8 mL (Y/P) Yellow = BIO Purple = DIG
HKB18003 HKD38003	HPV typing	40	HPV 6/11 DNA probe: size: 100-300 bp, vector: pSP 3.0 kb, region: total genome 7.8 kb HPV type 6 and 7.9 kb HPV type 11 HPV 16/18 DNA probe: size: 100-300 bp, vector: pSP 3.0 kb, region: total genome 7.9 kb HPV type 16 and 7.9 kb HPV type 18 HPV 31/33 DNA probe: size: 100-300 bp, vector: pBR322 4.3 kb and a modified pSP ≈ 4.0 kb, region: total genome 7.9 kb HPV type 31 and 7.9 kb HPV type 33	3 x 0.8 mL (Y/P) Yellow = BIO Purple = DIG
HKB18047 HKD38047	CMV	40	CMV DNA probe: size: 100-300 bp, region: isolated total genome	1 x 0.8 mL (Y/P) Yellow = BIO Purple = DIG
HKB18049 HKD38049	Epstein-Barr	40	EBV DNA probe: size: 100-300 bp, vector: pDR720 4.0 kb, region: 4.2 kb (W fragment)	1 x 0.8 mL (Y/P) Yellow = BIO Purple = DIG
HKB18056 HKD38056	Herpes simplex	40	HSV 1/2 DNA probe: size: 100-300 bp, vector: pSPM 2.6 kb, region: three HSV2 Sma I fragments (total ≈ 3.0 Kb)*	2 x 0.8 mL (Y/P) Yellow = BIO Purple = DIG

All kits contain a positive and negative control probe:

- DISH positive control oligo probe: mixture of six 30-mer oligonucleotides complementary to ALU repeat.
- DISH negative control DNA probe: size: 100-300 bp, vector: pSP 3.0 kb.

*The HSV 1/2 probe provided with this kit stains both HSV 1 and HSV 2. In order to distinguish between HSV 1 and HSV 2, a high stringency wash ([formamid] > 60%; not provided with this kit) may be applied on consecutive sections. The high stringency wash results in a slightly weaker staining for HSV 2 and a much weaker staining for HSV 1 when compared to results obtained with the normal in this manual described procedure. Interpretation of results are solely the responsibility of the researcher.