

REMBRANDT®

In situ Hybridisation and Detection

Universal DISH & AP Detection Kit-v5

Biotin Label product code	Digoxigenin label product code	# Assays
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PanPath
Science Park Amsterdam
Kruislaan 406
1098 SM Amst erdam
The Netherlands
Tel. +31 20 751 2030
Fax +31 20 751 2031
E-mail info@panpath.nl
Web site www.panpath.nl

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Chapter 1 Introduction

1.1 Intended use

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

1.2 The ISH principle

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

1.3 Controls

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

1.4 Contents of a REMBRANDT® Universal DISH & AP Detection Kit

Item label	description	Item (cap) colour	Item contents description	Item amount
DIGEST	PEPSIN POW	Black vial	: Pepsin digestion reagent	1 gram
DIGEST	PEPSIN DIL	Transparent vial	: Pepsin diluent (1N HCl solution)	15 mL
PROBE	+ ¹ DISH	Pink vial	: DISH positive control oligo probe (BIO or DIG)	0.8 mL
PROBE	- ¹ DISH	Green vial	: DISH negative control DNA probe(BIO or DIG)	0.8 mL
PANWASH	GC% ¹ 50%	White vial	: PanWash (differentiation reagent)	2 x 15 mL
CONJ ¹ AP	Red vial	: AP-conjugated anti-DIG or anti-BIO	15 mL
SUBS	NBT/BCIP	Blue vial	: NBT/BCIP substrate	15 mL
COUNT	NFR	Orange vial	: Nuclear Fast Red counterstain	15 mL
WASH	TBS	White pouches	: TBS buffer salt	2 pcs

~ Kit contents continued ~

Item label	description	Item (cap) colour	Item contents description	Item amount
SUPPORT	GL SLIDES	White box A	: Teflon coated glass slides, double wells	50 pcs
SUPPORT	COVERSL	White box B	: Coverslips	100 pcs

1 Depends on Kit specification

1.5 Materials required but not included

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

1.6 Storage and shelf life

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

1.7 Safety precautions

- Some reagents contain 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, rinse with large volumes of clean water.
- Never pipette solutions by mouth.
- The control slide in the kit contains pathogenic material fixed in 4% para-formaldehyde making specimens non-infectious; however, we advise taking standard precaution measures for handling infectious organisms.

Item label	description	Item (cap) colour	Item contents description	Item amount
SUPPORT	GL SLIDES	White box A	: Teflon coated glass slides, double wells	50 pcs
SUPPORT	COVERSL	White box B	: Coverslips	100 pcs

1 Depends on Kit specification

Item label	description	Item (cap) colour	Item contents description	Item amount
SUPPORT	GL SLIDES	White box A	: Teflon coated glass slides, double wells	50 pcs
SUPPORT	COVERSL	White box B	: Coverslips	100 pcs

1 Depends on Kit specification

HANDLEIDING-V4 UNIVERSAL DISH-AP

VIAL-LABEL

METODICA D'USO-V4 UNIVERSAL DISH-AP

- SLIDEN ENPLAKKEN VAN PARAFFINE COUPES**
1. Snij 4-6 µm coupes en plak ze op voorbehandelde objectglazen
 2. Verwarm de glazen
 3. Deparaffineer in verse xylol
 4. Spoel in 100% ethanol en laat de glazen luchtdrogen

INCUBATIE TIJDEN

2-16 uur bij 56-60°C
2 x 10 min.
5 min.

DIGEST PEP SIN DIL

DIGEST PEP SIN POW

- PRETRATTAMENTO DELLE SEZIONI IN PARAFFINA**
1. Tagliare sezioni di 4-6 µm e depositare su vetrino italiano
 2. Scaldare i vetrini
 3. Togliere la paraffina usando xilolo fresco
 4. Sottrarre i vetrini in etanolo 100% ed asciugare all'aria

TEMPI DI INCUBAZIONE

2-16 ore a 56-60°C
2 x 10 min.
5 min.

PROTEOLITISCHE VOORBEHANDELING

1. Los het pepsine poeder (vriesd) in 4 ml gedestilleerdgedeioniseerd water op, verdeel in 150 µL porties en bewaar bij 20°C.

INCUBATIE TIJDEN

2-16 uur bij 56-60°C
2 x 10 min.
5 min.

DIGEST PEP SIN DIL

DIGEST PEP SIN POW

- TRATTAMENTO PROTETIVO**
1. Sequenziare le sezioni del kit, sciogliere la paraffina in polvere (fresca) in 4 ml di acqua distillata/deionizzata, quindi preparare e diluire di 150 µL e congelare a 20°C
 2. Diluire la pepsina 1N HCl (riserpagente) diluente siero a raggiungere la concentrazione richiesta dall'applicazione (Paraffinelli, Citologia o Compilar, vedasi punto 3)
 3. Diluire la soluzione proteolitica scongelata in HCl diluito secondo lo schema seguente (incubare ogni sezione con 300-400 µL):
Paraffinelli: 100x in 0,1N HCl;
Citologia: 25.000x in 0,01N HCl;
Compilar: 4 µL a 100 mL 0,01N HCl
Concella: 50.000x in 0,01N HCl
 4. Gellare l'eccezione della soluzione proteolitica
 5. Disidratare le sezioni in etanolo puro ed asciugare all'aria

TEMPI DI INCUBAZIONE

2-16 ore a 56-60°C
2 x 10 min.
5 min.

2. Verdun bij 1N HCl pepsine oplosmiddel (riserpagente) tot de concentratie die voor de applicatie nodig is (paraffinelli, cytologia of vries coupe zie 3)
3. Verdun de proteolytische stock oplossing in het verdunde HCl
en incubeer elk preparaat met 300-400 µL:
Paraffinelli: 100x in 0,1N HCl;
voeg 50 µL aan 5 mL 0,1N HCl toe
Citologia: 25.000x in 0,01N HCl;
voeg 4 µL aan 100 mL 0,01N HCl toe
Concella: 50.000x in 0,01N HCl;
voeg 2 µL aan 100 mL 0,01N HCl toe
4. Overmaat van de proteolytische werkoplossing weggoeden (alrijp vers bereiden)
5. Ontvaat de preparaten in opdrogende alcoholreeks en luchtdrogen

INCUBATIE TIJDEN

2-16 uur bij 56-60°C
2 x 10 min.
5 min.

DIGEST PEP SIN DIL

DIGEST PEP SIN POW

- PROCEDIMENTO DI IRRADIAZIONE**
1. Aggiungere 1 goccia (20 µl) di soluzione "prober" su ogni sezione.
 2. Denaturare.
 3. Iridizzare.
 4. Togliere il copri vetrino scaldando il vetrino in lampone TBS
 5. Aggiungere 50 goccie di PanWash (bianco) su ogni sezione
tramite subcontrollo positivo
 6. Lavare i vetri in lampone TBS

TEMPI DI INCUBAZIONE

5 min. subbocco riscaldante a 95°C
16 ore a 37°C in incubatrice
10 min.
15 min. subbocco riscaldante a 37°C
3 x 1 min.

- HYDRISATIE PROCEDURE**
1. Incubeer elk preparaat met probe reagens: 1 druppel of 20 µl endectaf met dekglasje
 2. Denatureer
 3. Iridificeer
 4. Verwijder dekglasje door preparaten in TBS buffer te dempen
 5. Incubeer elk preparaat met PanWash (wit): 50 druppels met uitwisseling van de positieve controle
 6. Spoel alle preparaten in TBS buffer

INCUBATIE TIJDEN

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

DIGEST PEP SIN DIL

DIGEST PEP SIN POW

- PROCEDIMENTO DI DETEZIONE E COLORAZIONE**
1. Aggiungere ad ogni sezione 2,3 goccie di coniugato (rosso)
 2. Sottrarre i vetrini in lampone TBS
 3. Sottrarre i vetrini in acqua distillata/deionizzata
 4. Aggiungere ad ogni sezione 2,3 goccie di substrato NBT/BCIP (blu) e incubare al buio
 5. Togliere l'eccesso di soluzione substrato e lavare i vetrini in acqua distillata/deionizzata
 6. **Controllare**: aggiungere ad ogni sezione 2,3 goccie di "counterstain" (granatino)
 7. Lavare le sezioni in acqua distillata/deionizzata
 8. Preparare le sezioni per l'osservazione al microscopio

TEMPI DI INCUBAZIONE

5 min. subbocco riscaldante a 95°C
16 ore a 37°C in incubatrice
10 min.
15 min. subbocco riscaldante a 37°C
3 x 1 min.

- DETECTIE EN TEGENKLEURINGSPROCEDURE**
1. Incubeer elk preparaat met coniugaat (rood): 2,3 druppels
 2. Spoel de preparaten met TBS buffer
 3. Spoel de preparaten met gedestilleerdgedeioniseerd water
 4. Incubeer elk preparaat met NBT/BCIP substraat (blauw): 2,3 druppels en incubeer in donker
 5. Spoel de preparaten met gedestilleerdgedeioniseerd water
 6. **Controlleren**: incubeer elk preparaat met tegenkleuring (oranje): 2,3 druppels
 7. Spoel de preparaten met gedestilleerdgedeioniseerd water
 8. Dekdecoups af

INCUBATIE TIJDEN

30 min. bij 37°C hele plaat
3 x 1 min.
1 min.
5-15 min. bij 37°C hele plaat
3 x 1 min.
1 min.
3 x 1 min.

DIGEST PEP SIN DIL

DIGEST PEP SIN POW

- PROCEDIMENTO DI DETEZIONE E COLORAZIONE**
1. Aggiungere ad ogni sezione 2,3 goccie di coniugato (rosso)
 2. Sottrarre i vetrini in lampone TBS
 3. Sottrarre i vetrini in acqua distillata/deionizzata
 4. Aggiungere ad ogni sezione 2,3 goccie di substrato NBT/BCIP (blu) e incubare al buio
 5. Togliere l'eccesso di soluzione substrato e lavare i vetrini in acqua distillata/deionizzata
 6. **Controllare**: aggiungere ad ogni sezione 2,3 goccie di "counterstain" (granatino)
 7. Lavare le sezioni in acqua distillata/deionizzata
 8. Preparare le sezioni per l'osservazione al microscopio

TEMPI DI INCUBAZIONE

30 min. subbocco riscaldante a 37°C
3 x 1 min.
1 min.
5-15 min. subbocco riscaldante a 37°C
3 x 1 min.

1. Distribuir 2,3 gotitas de conjugado (cople) per specimen
2. Inmersionar las lames dans le tampon TBS
3. Inmersionar las lames dans le veau distillee ou deionisee
4. Agitar 2-3 gotitas du substrat NBT/BCIP (bleu) a chaque echantillon et rinculer dans l'obscurlite
5. Rincer les lames avec de veau distillee ou deionisee
6. **Quilancer** agitar 2-3 gotitas de la solution colorante (orange)
7. Rincer les lames avec de veau distillee ou deionisee
8. Montrer les sections et examiner les lames au microscope

1. Distribuir 2,3 gotitas de conjugado (cople) per specimen
2. Inmersionar las lames dans le tampon TBS
3. Inmersionar las lames dans le veau distillee ou deionisee
4. Agitar 2-3 gotitas du substrat NBT/BCIP (bleu) a chaque echantillon et rinculer dans l'obscurlite
5. Rincer les lames avec de veau distillee ou deionisee
6. **Quilancer** agitar 2-3 gotitas de la solution colorante (orange)
7. Rincer les lames avec de veau distillee ou deionisee
8. Montrer les sections et examiner les lames au microscope

30 min, 37°C bloc chauffant	30 min, 37°C bloc chauffant
3 x 1 min	3 x 1 min
1 min	1 min
5-15 min, 37°C bloc chauffant	5-15 min, 37°C bloc chauffant
3 x 1 min	3 x 1 min
1 min	1 min
3 x 1 min	3 x 1 min

30 min, 37°C bloc chauffant	30 min, 37°C bloc chauffant
3 x 1 min	3 x 1 min
1 min	1 min
5-15 min, 37°C bloc chauffant	5-15 min, 37°C bloc chauffant
3 x 1 min	3 x 1 min
1 min	1 min
3 x 1 min	3 x 1 min



1.8 Performance precautions

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

1.9 Preparation of reagents in advance

Pepsin digestion reagent:
Dissolve the proteolytic reagent (black) in 4 mL of distilled or deionised water (upon receipt of the kit). Aliquot in portions of i.e.50 µL and store at -20°C.

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

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

1.10 Preparation of the proteolytic work solution

Prepare proteolytic work solution; 300 to 400 µL per section of 1 cm². 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.

GUIDE REFERENCE V4 UNIVERSAL DSH-AP

1. Preparer des sections
2. Charitez les lames
3. Desparaffinez dans du xylene frais
4. Immergez dans de l'ethanol 100% (ou ethanol absolu) et laissez secher a l'air

2-16 hrs à 56-60°C	2 x 10 min
5 min.	

VIAL LABEL

PREST	PEPSIN 100X
PREST	PEPSIN 1N

GUIA DE REFERENCIA V4 UNIVERSAL DSH-AP

1. Preparar cortes de 4-6 µm y depositarlas sobre portales tratados
2. Charitar las portales
3. Desparafinar en xileno limpio
4. Sumergir los portales en etanol absoluto y secar al aire

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



Chapter 2 REMBRANDT® Universal DISH & AP Detection Protocol

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

2.1 Specimen collection and pre-treatment

Paraffin embedded tissue sections

A standard procedure for tissue fixation and embedding usually involves the use of formalin and paraffin. The optimal tissue block size is 0.5 cm³. The formalin should be buffered and fixation times should (preferably) not exceed 12 hours. Excess and/or insufficient fixation may yield suboptimal morphology and target preservation. Embedding in paraffin should not exceed temperatures of 65°C.

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

Cytological specimens

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

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

Frozen sections

In general, small pieces of tissue (max. 1 cm³) are snap frozen in liquid nitrogen and either stored at -70°C or used immediately. Frozen sections are more fragile than paraffin embedded tissue sections. They should be handled with care and processed as soon as possible.

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

2.2 Proteolytic treatment

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



	PREPARATION OF PAPER-FIN SECTIONS	INCUBATION TIME	WAL-LABEL	ANLEITUNG VON UNIVERSAL DISH-AP	INKUBATIONSZEITEN
	1. Cut 4µm sections and collect on treated glass slides	2-16 hrs at 56-60°C		1. 4µm Schritte anfertigen und auf vorbereitete Objektträger ziehen	2-16 Stunden bei 56-60°C
	2. Heat slides	2 x 10 min.		2. Schritte hitzen	2 x 10 Min.
	3. Dewax in fresh xylene	5 min.		3. in Xylolentparaffinen	5 Min.
	4. Soak slides in 100% ethanol and air dry			4. Schritte in 100% Ethanol entwässern und lufttrocknen	
	PROTEOLYTIC TREATMENT			PROTEOLYTISCHE BEHANDLUNG	
	1. 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		FINDEST - PEP SIN - POW	1. Pepsin Pulver (Schwarz) in 4ml destilliertem/deionisiertem Wasser lösen. In 150 µL Portionen aliquotieren und bei -20°C aufbewahren.	
	2. Dilute the 11N HCl pepsin solution (enzym) to the application required concentration (green, yellow or brown; see 3)		FINDEST - PEP SIN - LIQ	2. 11N HCl Pepsin -Lösungsmittel (Transparenz) auf die Konzentration verdünnen, die für die entsprechende Anwendung notwendig ist (Paraffinschnitt, Zytologisches Präparat oder Gefäßschnitt; siehe 3)	
	3. Dilute thawed proteolytic stock solution in diluted HCl and incubate each specimen with 300-400 µL			3. Verdünne die aufgetaute proteolytische Lösung in verdünntem HCl; jedes Präparat mit 300-400 µL inkubieren.	
	paraffin: 100x in 0.1N HCl; add 50 µl to 5 ml 0.1N HCl; cytological: 25.000x in 0.01N HCl; add 4µl to 100 ml 0.01N HCl; frozen: 50.000x in 0.01N HCl; add 2µl to 100 ml 0.01N HCl	30 min, 37°C heating block 10 min, 37°C heating block 10 min, 37°C heating block		Paraffinschnitt: 100x in 0.1N HCl; 50 µL proteolytische Lösung zu 5 ml 0.1N HCl geben Zytologisches Präparat: 25.000x in 0.01N HCl; 4µL proteolytische Lösung zu 100 ml 0.01N HCl; geben Gefäßschnitt: 50.000x in 0.01N HCl; 2 µL proteolytische Lösung zu 100 ml 0.01N HCl; geben	30 Min. bei 37°C Heizplatte 10 Min bei 37°C Heizplatte 10 Min bei 37°C Heizplatte
	4. Discard excess proteolytic work solution	10 min, 37°C heating block		2. µL proteolytische Lösung zu 100 ml 0.01N HCl; geben	10 Min. bei 37°C Heizplatte
	5. Dehydrate slides in graded ethanol and air dry	3 x 1 min.		4. Überschüssige proteolytische Lösung entfernen 5. Schritte in Ethanolreihe entwässern und lufttrocknen	3 x 1 Min.
	HYBRIDIZATION PROCEDURE			HYBRIDISIERUNGSPROZEDUR	
	1. Apply 1 drop or 20 µl of probe solution per specimen; cover with coverslip			1. 1 Tropfen oder 20 µl der Sonde auf jedes Präparat geben und mit einem Deckglas abdecken	
	2. Denature			2. Denaturieren	
	3. Hybridize	5 min, 95°C heating block 16 hrs 37°C incubator		3. Hybridisieren	5 Min. bei 95°C Heizplatte 16 Stunden bei 37°C Ofen
	4. Remove coverslips by soaking slides in TBS buffer	10 min, 37°C heating block	WASCH - TBS	4. Entfernen der Deckgläser durch Einweichen in TBS Puffer	10 Min.
	5. Apply 5-6 drops of Parawash (white) to each specimen except the positive control	15 min, 37°C heating block	PARAWASCH - GRÜN, ORANGE	5. 5-6 Tropfen Parawash (Weiß) zu jedem Präparat geben (mit Ausnahme der Positivkontrolle)	15 Min. bei 37°C Heizplatte 3 x 1 Min.
	6. Wash all slides in TBS buffer	3 x 1 min.	WASCH - TBS	6. Präparate in TBS Puffer spülen	3 x 1 Min.
	DETECTION AND STAINING PROCEDURE			DETEKTIONS- UND FÄRBEPROZEDUR	
	1. Apply 2-3 drops of the conjugate (red) to each specimen	30 min, 37°C heating block	COUL - AP	1. 2-3 Tropfen Konjugat (Rot) auf jedes Präparat geben	30 Min. bei 37°C Heizplatte
	2. Soak slides in TBS buffer	3 x 1 min.	WASCH - TBS	2. Präparate in TBS Puffer spülen	3 x 1 Min.
	3. Soak slides in distilled/deionized water	1 min.	PARAWASCH - GRÜN, ORANGE	3. Präparate mit destilliertem/deionisiertem Wasser spülen	1 Min.
	4. Apply 2-3 drops of NB1/B1CP substrate (blue) to each specimen and incubate in dark	5-15 min, 37°C heating block	SUBS - NITROBICP	4. 2-3 Tropfen des NB1/B1CP-Substrats (Blau) auf jedes Präparat geben Im dunkeln inkubieren	5-15 Min. bei 37°C Heizplatte
	5. Tap off excess substrate solution and wash slides in distilled/deionized water	3 x 1 min.	COUL - NITRO	5. Indestilliertem/deionisiertem Wasser spülen	3 x 1 Min.
	6. Optional: apply 2-3 drops of counterstain (orange) to each specimen	1 min.		6. Optional: 2-3 Tropfen der Gegenfärbelösung (Orange) auf jedes Präparat geben	1 Min.
	7. Wash slides in distilled/deionized water	3 x 1 min.		7. Indestilliertem/deionisiertem Wasser spülen	3 x 1 Min.
	8. Mount sections for microscopic evaluation			8. Präparate für die mikroskopische Beurteilung abdecken	



6.5 Positive staining of the negative control

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

6.6 Non-specific background staining

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

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

6.7 Cross Hybridisation

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

2.3 Hybridisation procedure*Denaturation and Hybridisation*

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

Differentiation (stringency wash) and rinsing

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

2.4 Detection and staining procedure

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

Take the slides out, wipe off excess of water and dry around the edges using a lint-free cloth. Ensure that the specimen on the slide is not disrupted. Apply 2-3 drops of NBT/BCIP substrate (blue) to each specimen and transfer the slides onto a 37°C heating block or slide warmer. Incubate in the dark for 5-15 minutes at 37°C (examine the colour development every 5 minutes microscopically). Tap off excess substrate solution and rinse the slides for 3x 1 minute in changes of distilled or deionised water. The slides are now ready to be mounted or counterstained.

2.5 Counterstain procedure

When a contrast colour is desired, the slides can be counterstained using 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 rinse the slides briefly in distilled or deionised water. Mount the slides by using an aqueous mounting medium. Interpret the results under the microscope.

Chapter 3 Limitations of Procedure

3.1 Limitations

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

3.2 Interpretation of the results

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

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

6.3 Weak or no staining on a suspected positive sample

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

6.4 Negative staining of the positive control

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

Chapter 6 Trouble Shooting Guide

6.1 Introduction

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

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

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

6.2 No section or cells left on the slides

Possible causes	Remedies
■ Sample preparation.	→ Make sure samples are prepared according to protocol, the tissue is fixed in neutral buffered formalin and the slides are air dried well.
■ Tissue section too thin.	→ Optimal thickness of the tissue is 4-6 µm.
■ Wrong (side of) glass slide used.	→ Use only organosilane coated glass slides.
■ Pepsin concentration too high.	→ Make sure correct concentration of pepsin is used (depending on type of specimen).
■ Digestion step too long.	→ Reduce digestion time (15 minutes instead of 30 minutes) or digest at room temperature.
■ Denaturation.	→ Make sure temperature is 95 ± 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.

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

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

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

Use high power magnification to see whether:

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

Product in combination with other devices

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

Specifications of the DNA probes:

	Positive Control DNA	Negative Control DNA
Specificity	100%	100%
Sensitivity	95%	95%

Chapter 4 References

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Chapter 5 Probe specifications

REMBRANDT® Biotin¹ and Digoxigenin² labelled DNA control probe specifications

PRODUCT CODE	LABEL	DNA PROBE SPECIFICATIONS			
		Description	Size	Region	Vector
Q001P.0100 Q001P.9900	BIO DIG	Negative control probe for DNA (CONTROL – xxx DISH)*	100-300 bp		pSP; 3.0 Kb
Q151P.0100 Q151P.9900	BIO DIG	Positive control probe for DNA (CONTROL + xxx DISH)*	30-mer oligonucleotide	Mixture of six oligonucleotides complimentary to ALU repeats	

* xxx = label (BIO or DIG)

Contents : - clear vial, yellow cap = BIO labelled probe; 0.8 mL (25-40 assays)
 - clear vial, purple cap = DIG labelled probe; 0.8 mL (25-40 assays)
 - white vial, white cap = matching PanWash; 15 mL
 R013R.0000 for probes with GC % < 50%
 R014R.0000 for probes with GC % > 50%

Format : ready to use
 Application : colorimetric detection of respective DNA in human specimen by *in situ* hybridisation (ISH)
 Purification : by size exclusion chromatography
 Detection limit : 3-10 pg by filter hybridisation
 Storage : refrigerated (2-8 °C); do not freeze
 Stability : until expiry date printed on label
 Precautions : - homogenise solutions before use
 - avoid contact with eyes and skin; do not swallow

1 The probes in this product are labelled with the Universal Linkage System (ULS™). 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.

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