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REMBRANDT®

In situ Hybridisation and Detection

Universal RISH & AP Detection Kit-v5

Biotin Label product code	Digoxigenin label product code	# Assays
A000K.0105	A000K.9905	100

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KIT MANUAL REMBRANDT® UNIVERSAL RISH & AP DETECTION

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 RNA 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 RISH & AP Dectection 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] RISH	Pink vial	: RISH positive control oligo probe (BIO or DIG)	0.8 mL
PROBE	-| .....[1] RISH	Green vial	: RISH negative control oligo probe(BIO or DIG)	0.8 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.

HANDLEIDING-v4 UNIVERSAL RISH-AP

SNIJDEN VAN PLAKKEN VAN PARAFFINE COUPES
1. Snij 4-6 µm coupes en plak ze op voorbehandelde objectglaasjes
2. Verwarm de glaasjes
3. Deparaffineer in verse xylol
4. Spoel in 100% ethanol en laat de glaasjes luchtdrogen

PROTEOLYTISCHE VOORBEHANDELING (RNASE VRIJ)
1. Los het pepsine poeder (zwart) in 4 ml. gedestilleerd/gedeioniseerd water op, verdeel in 150 µL porties en bewaar bij -20 °C.
2. Verdun het 1N HCl pepsine oplosmiddel (transparant) tot de concentratie die voor de applicatie nodig is (paraffine, cytologie of vries coupe: zie 3)
3. Verdun de proteolytische stock oplossing in het verdunde HCl en incubeer elk preparaat met 300-400 µL:
   paraffine 100x in 0.1N HCl:
   voeg 50 µL aan 5 mL 0.1N HCl toe
   cytologie 25.000x in 0.01N HCl:
   voeg 4 µL aan 100 mL 0.01N HCl toe
   vries 50.000x in 0.01N HCl:
   voeg 2 µL aan 100 mL 0.01N HCl toe
4. Overmaat van de proteolytische werkoplossing weggooien (altijd vers bereiden)
5. Ontwater de preparaten in oplopende alcoholreeks en luchtdrogen

HYBRIDISATIE PROCEDURE (RNASE VRIJ)
1. Incubeer elk preparaat met probe reagens: 1 druppel of 20 µl en dek af met dekglaasje
2. Hybridiseer
3. Verwijder dekglaasje door preparaten in TBS buffer te dompelen
4. Spoel alle preparaten in TBS buffer

DETECTIE EN TEGENKLEURINGSPROCEDURE
1. Incubeer elk preparaat met conjugaat (rood): 2-3 druppels
2. Spoel de preparaten met TBS buffer
3. Spoel de preparaten met gedestilleerd/gedeioniseerd water
4. Incubeer elk preparaat met NBT/BCIP substraat (blauw): 2-3 druppels en incubeer in donker
5. Spoel de preparaten met gedestilleerd/gedeioniseerd water
6. Optioneel: incubeer elk preparaat met tegenkleuring (oranje): 2-3 druppels
7. Spoel de preparaten met gedestilleerd/gedeioniseerd water
8. Dek de coupes af

VIAL-LABEL

	DIGEST	PEPSIN_ROW
	DIGEST	PEPSIN_DIL

	WASH	TBS
	WASH	TBS

	CONJ	..... AP
	WASH	TBS

SUBS	NBT/BCIP

COUNT	NFR

METODICA D'USO-v4 UNIVERSAL RISH-AP

INCUBATIE TIJDEN

2-16 uur bij 56-60°C	
2 x 10 min.	
5 min.	
30 min. bij 37°C hete plaat	
16 uur bij 37°C stoof	
10 min.	
3 x 1 min.	
10 min. bij 37°C hete plaat	
3 x 1 min.	
30 min. bij 37°C hete plaat	
3 x 1 min.	
1 min.	
5-15 min. bij 37°C hete plaat	
3 x 1 min.	
1 min.	
3 x 1 min.	

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

TRATTAMENTO PROTEOLITICO (PRIVO DI RANASE)
1. Seguendo le istruzioni del kit, sciogliere la pepsina in polvere (nera) in 4 mL di acqua distillata/deionizzata, quindi preparare aliquote di 150 µL e congelare a -20°C.
2. Diluire la pepsina 1N HCl (trasparente) diluente sino a raggiungere la concentrazione richiesta dall'applicazione (Paraffinati, Citologici o Congelati: vedasi punto 3)
3. Diluire la soluzione proteolitica scongelata in HCl diluito secondo lo schema seguente (Incubare ogni sezione con 300 -400 µL):
   Paraffinati 100x in 0.1N HCl:
   aggiungere 50 µL a 5 mL 0.1N HCl
   Citologici: 25'000x in 0.01N HCl:
   aggiungere 4 µL a 100 mL 0.01N HCl
   Congelati: 50'000x in 0.01N HCl:
   aggiungere 2 µL a 100 mL 0.01N HCl
4. Gettare l'eccedenza della soluzione proteolitica
5. Disidratare le sezioni in etanolo puro ed asciugare all'aria

PROCEDIMENTO DI IBRIDAZIONE (PRIVO DI RANASE)
1. Aggiungere 1 goccia o 20 µl di soluzione "probe" su ogni sezione. Coprire con coprivetrino.
2. Ibridizzare
3. Togliere il coprivetrino sciacquando il vetrino in tampone TBS
4. Lavare tutti i vetrini in tampone TBS

PROCEDIMENTO DI DETEZIONE E COLORAZIONE
1. Aggiungere ad ogni sezione 2-3 gocce di coniugato (rosso)
2. Sciacquare i vetrini in tampone TBS
3. Sciacquare i vetrini in acqua distillata/deionizzata
4. Aggiungere ad ogni sezione 2-3 gocce 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. Opzionale: aggiungere ad ogni sezione 2-3 gocce di "counterstain" (arancione)
7. Lavare le sezioni in acqua distillata/deionizzata
8. Preparare le sezioni per l'osservazione al microscopio

30 min. su blocco riscaldante a 37°C	
10 min. su blocco riscaldante a 37°C	
10 min. su blocco riscaldante a 37°C	
3 x 1 min.	
16 ore a 37°C in incubatrice	
10 min.	
3 x 1 min.	
30 min. su blocco riscaldante a 37°C	
3 x 1 min.	
1 min.	
5-15 min. su blocco riscaldante a 37°C	
3 x 1 min.	
1 min.	
3 x 1 min.	

GUIDE RÉFÉRENCE-v4 UNIVERSAL RISH-AP

	TEMPS D'INCUBATION
PRE-TRAITEMENT DES SECTIONS PARAFFINEES	
1. Préparez des sections de 4-6 µm et coller les en lames traitées	
2. Chauffez les lames	2-16 hrs à 56-60°C
3. Deparaffinez dans du xylène frais	2 x 10 min.
4. Immergez dans de l'éthanol 100% (ou ethanol absolu) et laissez sécher à l'air	5 min.
TRAITEMENT PROTEOLYTIQUE (SANS ARNASE)	
1. Dissolvez la poudre de pepsine (noire) dans 4 ml d'eau distillée ou deionnisée, divisez la solution en aliquotes de 150 µl et congelez les (-20°C).	
2. Diluez la solution HCl 1N (solution de pepsine en tant que diluant, transparent) selon l'usage préféré (paraffiné, cytologique ou congelé)	
3. Diluez une aliquote de la solution du stock proteolytique avec la solution diluée de HCl et incubez chaque échantillon dans 300-400 µl de la manière suivante:	
dilution paraffinée: 100x dans 0.1N HCl; ajouter 50 µl à 5 ml de HCl 0.01N	30 min. 37°C bloc chauffant
dilution cytologique 25,000x dans 0.01N HCl; ajouter 4 µl à 100 ml HCl 0.01N	10 min. 37°C bloc chauffant
dilution congelée 50,000x dans 0.01N HCl; ajouter 2 µl à 100 ml de HCl 0.01N	10 min. 37°C bloc chauffant
4. Rincez avec de l'eau distillée ou deionisée	
5. Desfydratez les lames dans une série d'éthanol et laissez sécher à l'air	3 x 1 min.
PROTOCOLO HYBRIDATION (SANS ARNASE)	
1. Ajoutez une goutte ou 20 µl d'une solution de sonde par échantillon et couvrez avec une lamelle	
2. Hybridez	16 hrs 37°C incubateur
3. Diffusez verticalement le tampon TBS sur les lamelles	10 min.
4. Rincez toutes les lames avec le tampon TBS	3 x 1 min.
PROTOCOLE DE DETECTION ET DE COLORATION	
1. Distribuez 23 gouttes de conjugart (rouge) par spécimen	30 min. 37°C bloc chauffant
2. Immergez les lames dans le tampon TBS	3 x 1 min.
3. Immergez les lames dans de l'eau distillée ou deionisée	1 min.
4. Ajoutez 2-3 gouttes du substrat NBT/BCIP (bleu) à chaque échantillon et incubez dans l'obscurité	5-15 min. 37°C bloc chauffant
5. Rincez les lames avec de l'eau distillée ou deionisée	3 x 1 min.
6. Optionnel: ajoutez 2-3 gouttes de la solution colorante (orange)	1 min.
7. Rincez les lames avec de l'eau distillée ou deionisée	3 x 1 min.
8. Montez les sections et examinez les lames au microscope	

VIAL-LABEL

DIGEST	PEPSIN_BOUW
DIGEST	PEPSIN_DIL
WASH	TBS
WASH	TBS
CONJ	AP
WASH	TBS
SUBS	NBT/BCIP
COUNT	NFR

GUIA DE REFERENCIA-v4 UNIVERSAL RISH -AP

	TIEMPO DE INCUBATION
PRETRATAMIENTO DE LOS CORTES DE PARAFINA	
1. Preparar cortes de 4-6 µm y depositarlas sobre portas tratados	
2. Calentar los portas	2-16 horas A 56-60ºC
3. Desparafinar en xileno limpio	2 x 10 minutos
4. Sumergir los portas en etanol absoluto y secar al aire	5 minutos
TRATAMIENTO PROTEOLITICO (LIBRE DE RNASA)	
1. A la recepción del kit disolver la pepsina en polvo (vial negro) en 4 ml de agua destilada/desionizada, hacer alicuotas de 150 µl y congelar a -20ºC.	
2. Diluir el diluyente de la pepsina (vial transparente) C/H 1N a la concentración requerida para la aplicación (parafina, citológia o congelación; ver 3)	
3. Diluir la solución proteolitica stock descongelada en C/H diluído e incubar cada muestra con 300-400 µl:	
Parafina: 100X en C/H 0.1N; añadir 50 µl a 5 ml de C/H 0.1N	30 minutos en un termobloque a 37ºC
Citológia: 25.000X en C/H 0.01N ; añadir 4 µl a 100ml de C/H 0.01N	10 minutos en un termobloque a 37ºC
Congelación: 50,000X en C/H 0.01N; añadir 2 µl a 100 ml de C/H 0.01N	10 minutos en un termobloque a 37ºC
4. Eliminar el exceso de solución proteolítica a la dilución de trabajo	
5. Deshidratar los portas en soluciones alcohólicas crecientes y secar al aire	3 x 1 minuto
PROTOCOLO DE HIBRIDACION (LIBRE DE RNASA)	
1. Añadir 1 gota o 20 µl de la solución de la sonda por mue stra. Cubrir con un cubre.	
2. Hibridizar	16 horas a 37ºC en un incubador
3. Retirar los cubres sumergiendo los portas en tampón TBS	10 minutos
4. Lavar todos los portas en tampón TBS	3 x 1 minuto
PROTOCOLO DE DETECCION Y TINCION	
1. Añadir 2-3 gotas del conjugado (vial rojo) a cada muestra	30 minutos a 37ºC en un termobloque
2. Sumergir los portas en tampón TBS	3 x 1 minuto
3. Sumegir los portas en agua desionizada	1 minuto
4. Añadir 2-3 gotas del substrato NBT/BCIP (vial azul) a cada muestra e incubar en oscuridad	5-15 minutos a 37ºC en un termobloque
5. Eliminar el exceso de solución de substrato y lavar los portas en agua destilada o desionizada	3 x 1 minuto
6. Opcionalmente: añadir 2-3 gotas de solución de contraste (vial naranja) a cada muestra	1 minuto
7. Lavar los portas en agua desionizada	3 x 1 minuto
8. Montar los portas para su evaluación por microscopía	

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.
- Work Rnase-free directly after deparaffinisation until the hybridization step is completed.
- Wear gloves and treat glassware overnight at 200°C.
- ***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.

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 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.

Chapter 2 REMBRANDT® Universal RISH & 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 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 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 cm^3) are snap frozen in liquid nitrogen and either stored at -70°C or used immediately. Frozen sections are more fragile than paraffin embedded tissue sections. They should be handled with care and processed as soon as possible.

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

2.2 Proteolytic treatment (Rnase-free)

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.

REFERENCE GUIDE-v4 UNIVERSAL RISH-AP

	INCUBATION TIME		
PRETREATMENT OF PARAFFIN SECTIONS			
1. Cut 4-6 µm sections and collect on treated glass slides			
2. Heat slides	2-16 hrs 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 (RNASE FREE)			
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.		DIGEST	PEPSIN POW
2. Dilute the 1N HCl pepsin diluent (transparent) to the application required concentration (paraffin, cytological or frozen; see 3)		DIGEST	PEPSIN DIL
3. 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. 37°C heating block		
cytological: 25,000x in 0.01N HCl; add 4µl to 100 ml 0.01N HCl	10 min. 37°C heating block		
frozen: 50,000x in 0.01N HCl; add 2µl to 100 ml 0.01N HCl	10 min. 37°C heating block		
4. Discard excess proteolytic work solution			
5. Dehydrate slides in graded ethanol and air dry	3 x 1 min.		
HYBRIDIZATION PROCEDURE (RNASE FREE)			
1. Apply 1 drop or 20 µl of probe solution per specimen; cover with coverslip			
2. Hybridize	16 hrs 37°C incubator	WASH	TBS
3. Remove coverslips by soaking slides in TBS buffer	10 min.	WASH	TBS
4. Wash all slides in TBS buffer	3 x 1 min.	WASH	TBS
DETECTION AND STAINING PROCEDURE			
1. Apply 2-3 drops of the conjugate (red) to each specimen	30 min. 37°C heating block	CONJ	..... AP
2. Soak slides in TBS buffer	3 x 1 min.	WASH	TBS
3. Soak slides in distilled/deionized water	1 min.		
4. Apply 2-3 drops of NBT/BCIP substrate (blue) to each specimen and incubate in dark	5-15 min. 37 °C heating block	SUBS	NBT/BCIP
5. Tap off excess substrate solution and wash slides in distilled/deionized water	3 x 1 min.		
6. Optional: apply 2-3 drops of counterstain (orange) to each specimen	1 min.	COUNT	NFR
7. Wash slides in distilled/deionized water	3 x 1 min.		
8. Mount sections for microscopic evaluation			

VIAL-LABEL

ANLEITUNG-v4 UNIVERSAL RISH-AP

	INKUBATIONSZEITEN
HERSTELLUNG VON PRÄPARATSCHNITTEN	
1. 4-6 µm Schnitte anfertigen und auf vorbehandelte Objektträger ziehen	
2. Schnitte inkubieren	2-16 Stunden bei 56-60°C
3. in Xylol entparaffinieren	2 x 10 Min.
4. Schnitte in 100% Ethanol entwässern und lufttrocknen	5 Min.
PROTEOLYTISCHE BEHANDLUNG (RNASE FREI)	
1. Pepsin Pulver (Schwarz) in 4 mL destilliertem/deionisiertem Wasser lösen, in 150 µL Portionen aliquotieren und bei -20°C aufbewahren.	
2. 1N HCl Pepsin -Lösungsmittel (Transparent) auf die Konzentration verdünnen, die für die entsprechende Anwendung notwendig ist (Paraffinschnitt, Zytologisches Präparat oder Gefrierschnitt; siehe 3)	
3. Verdünne die aufgetaute proteolytische Lösung in verdünntem HCl; jedes Präparat mit 300-400 µL inkubieren.	
Paraffinschnitt: 100x in 0,1N HCl: 50 µL proteolytische Lösung zu 5 mL 0,1N HCl geben	30 Min. bei 37°C Heizplatte
Zytologisches Praeparat: 25.000x in 0,01N HCl: 4µL proteolytische Lösung zu 100 mL 0,01N HCl geben	10 Min bei 37°C Heizplatte
Gefrierschnitt: 50,000x in 0,01N HCl: 2 µL proteolytische Lösung zu 100 mL 0,01N HCl geben	10 Min. bei 37°C Heizplatte
4. Überschüssige proteolytische Lösung verwerfen	
5. Schnitte in Ethanolreihe entwässern und Lufttrocken	3 x 1 Min.
HYBRIDISIERUNGSPROZEDUR (RNASE FREI)	
1. 1 Tropfen oder 20 µl der Sonde auf jedes Präparat geben und mit einem Deckglas abdecken	
2. Hybridisieren	16 Stunden bei 37°C Ofen
3. Entfernen der Deckgläser durch Eintauchen in TBS Puffer	10 Min.
4. Präparate in TBS Puffer spülen	3 x 1 Min.
DETEKTIONS UND FÄRBEPROZEDUR	
1. 2-3 Tropfen Konjugat (Rot) auf jedes Präparat geben	30 Min. bei 37°C Heizplatte
2. Präparate in TBS Puffer spülen	3 x 1 Min.
3. Präparate mit destilliertem/deionisiertem Wasser spülen	1 Min.
4. 2-3 Tropfen des NBT/BCIP -Substrats (Blau) auf jedes Präparat geben im dunkeln inkubieren	5-15 Min. bei 37°C Heizplatte
5. In destilliertem/deionisiertem Wasser spülen	3 x 1 Min.
6. Optional: 2-3 Tropfen der Gegenfärbelösung (Orange) auf jedes Präparat geben	
7. In destilliertem/deionisiertem Wasser spülen	3 x 1 Min.
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 ± 2°C.
■ 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
■ Drying out of the section.	→ Incubate all procedure steps in a moisturised environment; prevent evaporation
■ 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 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 (Rnase-free)

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!). 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).

Washing

– Remove coverslips by submerging the slides in TBS buffer. Soak the slides until the coverslips fall off. Rinse the slides in TBS buffer for 10 minutes. Take the slides out, wipe off excess buffer and dry the edges using a lint-free cloth.

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 um 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 been 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 fix ation.	→ 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.
■ Interfering internal structures of probes.	→ In case of RISH procedures, denature sample at 60°C for 5 minutes before applying probe solution.
■ Hybridisation 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.	→ 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.
■ Interfering internal structures of probes.	→ In case of RISH procedures, denature sample at 60°C for 5 minutes before applying probe solution.
■ 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 I*n 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 RNA probes:

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

Chapter 4 References

1. *Autillo-Touati A. et al., HPV Typing by In Situ Hybridization on Cervical Cytologic Smears with ASCUS, Acta Cytologica, Vol. 42, p. 631-638, 1998.*
2. *Benkemoun A. et al., Evaluation of KREATECH In Situ Hybridization Kits for Detection of Human Papillomavirus DNA on Cervical Smears with "ASCUS", 3rd International Symposium "Impact of Cancer Biotechnology Diagnostic & Prognostic Indicators", Nice, France, October 1996. Accepted for publication in Cancer Detection and Prevention.*
3. *Botma H.J. et al., Differential In Situ Hybridization for Herpes Simplex Virus Typing in Routine Skin Biopsies, Journal of Virological Methods, Vol. 53, p. 37-45, 1995*
4. *Cooper K. et al., Human Papillomavirus DNA in Oesophageal Carcinomas in South Africa, Journal of Pathology, Vol. 175, p. 273-277, 1995.*
5. *Davidson B. et al., Angiogenesis in Uterine Cervical Intraepithelial Neoplasia and Squamous Cell Carcinoma: An Immunohistochemical Study, International Journal of Gynecological Pathology, Vol. 16, p. 335-338, 1997.*
6. *Davidson B. et al., CD44 Expression in Uterine Cervical Intraepithelial Neoplasia and Squamous Cell Carcinoma: An Immunohistochemical Study, European Journal of Gyneacology and Oncology, Vol. XIX, no. 1, p. 46-49, 1998.*
7. *Davidson B. et al., Inflammatory Response in Cervical Intraepithelial Neoplasia and Squamous Cell Carcinoma of the Uterine Cervix, Pathology Research and Practice, Vol. 193, p. 491-495, 1997.*
8. *Gómez F. et al., Diagnosis of Genital Infection Caused by Human Papillomavirus Using In Situ Hybridization: The Importance of the Size of the Biopsy Specimen, Journal of Clinical Pathology, Vol. 48, p. 57-58, 1995.*
9. *Jing X. et al., Detection of Epstein-Barr Virus DNA in Gastric Carcinoma with Lymphoid Stroma, Viral Immunology, Vol. 10, No. 1, p. 49-58, 1997.*
10. *Sugawara I. et al., Detection of a Helicobacter Pylori Gene Marker in Gastric Biopsy Samples by Non-Radioactive In Situ Hybridization, Acta Histochemica et Cytochemica, Vol. 28, No. 3, p. 263-267, 1995.*
11. *Van den Brink W. et al., Combined ß-Galactosidase and Immunogold/Silver Staining for Immunohistochemistry and DNA In Situ Hybridization, Journal of Histochemistry and Cytochemistry, Vol. 38, p. 325-329, 1990.*
12. *Yanai H. et al., Epstein-Barr Virus Infection in Non-Carcinomatous Gastric Epithelium, Journal of Pathology, Vol. 183, p. 293-298, 1997.*
13. *Yonezawa S. et al., MUC2 Gene Expression is Found in Non-invasive Tumors But Not in Invasive Tumors of the Pancreas and Liver: Its Close Relationship with Prognosis of the Patients, Human Pathology, Vol. 28, No. 3, p. 344-352, 1997.*
14. *Ziol M. et al., Virological and Biological Characteristics of Cervical Intraepithelial Neoplasia grade I with marked koilocytic Atypia, Human pathology, Vol 29, No. 10, p. 1068-1073, 1998.*
15. *Evans M. et al, Biotinyl-Tyramide-Based In Situ Hybridization Signal Patterns Distinguish Human Papllloma Virus Type and Grade of Cervical Intraepithelial Neoplasia, Mod Pathol 2002; 15(12):1339-1347*
16. *Hopman A. et al., Transition of high grade cervical intraepithelial neoplasia to micro-invasive carcinoma is characterized by integration of HPV 16/18 and numerical chromosome abnormalities, J Pathol 2004; 202:23-33*
17. *Hopman A. et al., Human papillomavirus integration: detection by in situ hybridization and potential clinical application, J Pathol 2004; 202:1-4*
18. *Hafkamp et al., A subset of head and neck squamous cell carcinomas exhibits integration of HPV 16/18 DNA and overexpression of $p16^{INK4A}$ and p53 in the absence of mutations in p53 exons 5-8, Int. J. Cancer; 107(3):394-400*

Chapter 5 Probe specifications

REMBRANDT® Biotin[1] and Digoxigenin[1,2] labelled DNA control probe specifications

CAT. NO.	LABEL	DNA PROBE SPECIFICATIONS		
		Description	Size	Region
Q101P.0100 Q101P.9900	BIO DIG	Negative control probe for RNA (CONTROL – xxx RISH)*	26-mer oligonucleotide	1 oligonucleotide
Q152P.0100 Q152P.9900	BIO DIG	Positive control probe for RNA (CONTROL + xxx RISH)*	37-mer oligonucleotide	1 oligonucleotide complementary to Poly-A

* 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)
Format	:	ready to use
Application	:	colorimetric detection of respective RNA in human specimen by *in situ* hybridisation (ISH)
Detection limit	:	10-30 pg by filter hybridisation
Storage	:	refrigerated (2-8 °C); do not freeze
Stability	:	until expiry date printed on label
Precautions	:	- it is important to work RNase free in the period between deparaffinisation and hybridisation; wear gloves and treat glassware overnight at 200°C before use - 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).