

REMBRANDT®

In situ Hybridisation and Detection

Universal DISH & HRP Detection Kit-v5

Biotin Label product code	Digoxigenin label product code	# Assays
A001K.0101	A001K.9901	100

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- DETECTIE EN TEGENKLEURINGSPROCEDURE**
1. Incubeer elk preparaat met conjugaat (roos) 2,3 druppels
 2. Spoel de preparaten met TBS buffer
 3. Spoel de preparaten met gedestilleerdgedesondeerd water
 4. Bereid AEC (blauw) werkplossing volgens onderstaande tabel
 5. Incubeer elk preparaat met AEC werkplossing 2,3 druppels en incubeer in donker
 6. Spoel de preparaten met gedestilleerdgedesondeerd water
 7. Optioneel: Incubeer elk preparaat met tegenkleuring (oranje) 2, 3 druppels
 8. Spoel de preparaten met gedestilleerdgedesondeerd water
 9. Dek-decups af

Item	label	description	Item	cap	colour	Item	contents	description	Item	amount
		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.								

Item	label	description	Item	cap	colour	Item	contents	description	Item	amount
		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.								

- PROCEDIMENTO DI DEFEZIONE E COLO RAZIONE**
1. Aggiungere ad ogni sezione 2,3 gocce di conjugaato (rosso)
 2. Scliacquare i vetrini in tampone TBS
 3. Scliacquare i vetrini in acqua distillata/desodorizzata
 4. Preparare la soluzione di lavoro dell'AEC (blau) come segue:

Vol. di sezione	Vol. di goccia di substrato AEC	Volume di tampone AEC
1-13	4	2 mL
14-26	8	4 mL
27-39	12	6 mL
40-52	16	8 mL
 5. Aggiungere ad ogni sezione 2,3 gocce di soluzione di lavoro AEC e incubare al buio
 6. Togliere l'eccesso di soluzione, sostituito e lavare i vetrini in acqua distillata/desodorizzata
 7. Opzionale: aggiungere ad ogni sezione 2,3 gocce di "counterstain" (arancione)
 8. Lavare le sezioni in acqua distillata/desodorizzata
 9. Preparare le sezioni per l'osservazione al microscopio

Item	label	description	Item	cap	colour	Item	contents	description	Item	amount
		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.								

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 AEC and Horseradish Peroxidase (HRP) 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 & HRP 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	HRP	Red	vial				HRP-conjugated anti-DIG or anti-BIO		15 mL
SUBS		AEC	Blue	vial				AEC substrate		2 mL
BUFF		AEC	Blue	vial				AEC buffer		15 mL
COUNT		MG	Orange	vial				Methyl Green counterstain		15 mL
WASH		TBS	White	pouches				TBS buffer salt		2 pcs



HANDELIJG V-4 UNIVERSAL DISH-HRP

VIAL-LABEL

METODICA D'USO-V4 UNIVERSAL DISH-HRP

- INCUBATIE TIJDEN**
1. Snij 4-6 µm coupes en plak ze op voorbehandelde objectglazen
 2. Verwarm de glazen
 3. Deparaffineer in verse xyol
 4. Spoel in 100% ethanol en laat de glazen luchtdrogen

Item	label	description	Item	cap	colour	Item	contents	description	Item	amount
		2-16 uur bij 56-60°C								
		2 x 10 min.								
		5 min.								

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

Item	label	description	Item	cap	colour	Item	contents	description	Item	amount
		30 min. su blocco riscaldante a 37°C								
		10 min. su blocco riscaldante 37°C								
		10 min. su blocco riscaldante 37°C								
		10 min. su blocco riscaldante a 37°C								
		3 x 1 min.								

- TRATTAMENTO PROTETTICO (PRIMO DI PANWASH)**
1. Seguire le istruzioni del kit, sciogliere la pepsina in polvere (nera) in 4 mL di acqua distillata/desodorizzata, quindi preparare aliquoti di 150 µL e congelare a -20°C
 2. Diluire la pepsina 1N HCl (trasparente) diluente sino a raggiungere la concentrazione richiesta dall'applicazione (Prattina). Citologico
 3. Diluire la soluzione protettiva scongelata in HCl diluito secondo lo schema seguente (incubare ogni sezione con 300-400 µL):

Prattina	100% in 0.1N HCl	30 min. su blocco riscaldante a 37°C
aggiungere 50 µL	0.5 mL 0.1N HCl	10 min. su blocco riscaldante 37°C
aggiungere 4 µL	100 µL 0.01N HCl	10 min. su blocco riscaldante 37°C
aggiungere 500 µL	in 0.01N HCl	10 min. su blocco riscaldante a 37°C
aggiungere 7 µL	100 µL 0.01N HCl	3 x 1 min.
 4. Gettare l'eccesso della soluzione protettiva
 5. Disidratare le sezioni in etanolo puro ed asciugare all'aria

Item	label	description	Item	cap	colour	Item	contents	description	Item	amount
		5 min. su blocco riscaldante a 95°C								
		16 ore a 37°C in incubatore								
		10 min.								
		15 min. su blocco riscaldante a 37°C								
		3 x 1 min.								

- PROCEDIMENTO DI IRRADIAZIONE**
1. Aggiungere 1 goccia o 20 µL di soluzione "probe" su ogni sezione. Contre-contraintervino
 2. Denaturare
 3. Incubare
 4. Togliere la carpatina scaldando il vetrino in tampone TBS
 5. Aggiungere 5-6 gocce di Prattina (bianco) su ogni sezione
 6. Lavare sul controllo positivo
 7. Lavare tutti i vetrini in tampone TBS

TEMPERATURE DI INCUBAZIONE

~ 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.



PROTOCOL	DESCRIPTION	TEMPERATURE	TIME	REAGENT	CONCENTRATION	VOLUME
PRE-TREATMENT DES SECTIONS PARAFFINES	1. Préparer des sections de 4-6 µm et coller les en lames traitées		2-16 hrs à 56-60°C			
	2. Déparaffiner les lames		2 X 10 min.			
	3. Déparaffiner dans du xylène frais		5 min.			
	4. Immerger dans de l'éthanol 100% (ou éthanol absolu) et laissez sécher à l'air					
TRATAMIENTO PROTECTIVO	1. Dissolvez la poudre de pepsine (poudre) dans 4 ml d'eau distillée ou déionisée.					
	2. Diluez la solution HCl 1N (solution de pepsine en lani) que diluant (transparent)					
	3. Diluez la solution HCl 1N (solution de pepsine en lani) que diluant (transparent)					
PROTOCOL D'HYDRATION	1. Ajoutez une goutte ou 20 µl d'une solution de sonde par échantillon et ouvrez avec une lamelle		5 min. 95°C bloc chauffant			
	2. Déshydrat		16 hrs 37°C Incubateur			
	3. Diffusez verticalement le tampon TBS sur les lamelles		10 min. 37°C bloc chauffant			
	4. Ajoutez 50 gouttes de Pan Wash (blanc) à chaque échantillon		15 min. 37°C bloc chauffant			
	5. Rincez toutes les lames avec le tampon TBS		3 X 1 min.			
PROTOCOL DE DETECCION Y TINCION	1. Distribuez 23 gouttes de conjugué (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 déionisée		1 min.			
	4. Préparez la solution d'usage AEC (bleu) à partir du tableau ci-dessous:					
	5. Ajoutez 23 gouttes de la solution d'usage AEC par spécimen et incubez dans l'obscurité		5-15 min. 37°C bloc chauffant			
PRETRATAMIENTO DE LOS CORTES DE PARAFINA	1. Preparar cortes de 4-6 µm y depositarlos sobre portias tratadas		2-16 horas A 56-60°C			
	2. Calentar las portias		2 X 10 minutos			
	3. Desparafinar en xileno limpio		5 minutos			
	4. Sumergir los portias en etanol absoluto y secar al aire					
	5. Sumergir los portias en etanol absoluto y secar al aire					
PROTOCOL DE DETECCION Y TINCION	1. Añadir 23 gotas de conjugado (Vial rojo) a cada muestra		30 minutos en un termobloque a 37°C			
	2. Sumergir los portias en tampon TBS		3 X 1 minuto			
	3. Sumergir los portias en agua desionizada		1 minuto			
	4. Preparar el AEC a la solución de trabajo de acuerdo con la siguiente tabla:					
	5. Añadir 23 gotas de AEC a la solución de trabajo a cada muestra e incube en oscuridad		5-15 minutos a 37°C en un termobloque			



REFERENCE GUIDE v-4 UNIVERSAL DISH-HRP	INCUBATION TIME	VIAL LABEL	ANLEITUNG v-4 UNIVERSAL DISH-HRP																															
<p>PRETREATMENT OF PARAFFIN SECTIONS</p> <ol style="list-style-type: none"> Cut 46 µm sections and collect on treated glass slides Heat slides Dewax in fresh xylene Soak slides in 100% ethanol and/or dry <p>PROTEOLYTIC TREATMENT</p> <ol style="list-style-type: none"> 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 Dilute the 1N HCl peptidyl solution (transparent) to the application required concentration (paraffin, cytological or frozen; see 3) Dilute thawed proteolytic stock solution in diluted HCl and incubate each specimen with 300,400 µL: <ul style="list-style-type: none"> paraffin: 100x in 0.1N HCl add 50 µL to 5 mL 0.1N HCl cytological: 25,000x in 0.01N HCl add 4 µL to 100 mL 0.01N HCl add 2 µL to 100 mL 0.01N HCl add 50,000x in 0.01N HCl: <ul style="list-style-type: none"> add 2 µL to 100 mL 0.01N HCl Discard excess proteolytic work solution Dehydrate slides in graded ethanol and air dry <p>HYDRATATION PROCEDURE</p> <ol style="list-style-type: none"> Apply 1 drop or 20 µL of probe solution per specimen; cover with coverslip Denature Hydrate Remove coverslips by soaking slides in TBS buffer Apply 5-6 drops of PanWash (white) to each specimen except the positive control Wash all slides in TBS buffer <p>DETECTION AND STAINING PROCEDURE</p> <ol style="list-style-type: none"> Apply 2-3 drops of conjugate (red) to each specimen Soak slides in TBS buffer Soak slides in distilled/deionized water Prepare AEC (blue) work solution according the following table <table border="1"> <tr> <td>Multiplex Specimens</td> <td>Multiplex AEC substrate</td> <td>Vol. of AEC buffer</td> </tr> <tr> <td>1-13</td> <td>4</td> <td>2 mL</td> </tr> <tr> <td>14-26</td> <td>8</td> <td>4 mL</td> </tr> <tr> <td>27-39</td> <td>12</td> <td>6 mL</td> </tr> <tr> <td>40-52</td> <td>16</td> <td>8 mL</td> </tr> </table> <p>Tap off excess substrate solution and wash slides in distilled/deionized water</p> <ol style="list-style-type: none"> Optional: apply 2-3 drops of counterstain (orange) to each specimen Wash slides in distilled/deionized water Mount sections for microscopic evaluation 	Multiplex Specimens	Multiplex AEC substrate	Vol. of AEC buffer	1-13	4	2 mL	14-26	8	4 mL	27-39	12	6 mL	40-52	16	8 mL	<p>30 min. on a 37°C heating block</p> <p>16 hours at 37°C incubator</p> <p>10 min. on a 37°C heating block</p> <p>15 min. on a 37°C heating block</p> <p>3 x 1 min.</p>	<p>DIGEST PEPsin POWD</p> <p>DIGEST PEPsin DIL</p>	<p>HERSTELLUNG VON PARAFFINSCHNITTEN</p> <ol style="list-style-type: none"> 4-6 µm Schritte anfertigen und auf vorbereitete Objektträger ziehen Schritte inkludieren Xylolemparaffinieren Schritte in 100% Ethanol entwässern und lufttrocknen <p>PROTEOLYTISCHE BEHANDLUNG</p> <ol style="list-style-type: none"> Pepsin Pulver (Schwarz) in 4 mL destilliert/deionisiertem Wasser lösen; in 150 µL Portionen aliquotieren und bei -20°C aufbewahren 1N HCl Pepsin-Lösungsmittel (Transparenz) auf die Konzentrations verdünnen, die für die entsprechende Anwendung notwendig ist (Paraffinschnitt, Zytologisches Präparat oder Gefrierschnitt; siehe 3) Verdünnung 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 Zytologisches Präparat: 25,000x in 0.01N HCl 4 µL proteolytische Lösung zu 100 mL 0.01N HCl geben Färbesubstrat: 50,000x in 0.01N HCl 2 µL proteolytische Lösung zu 100 mL 0.01N HCl geben Überschüssige proteolytische Lösung verworfen Schritte in Ethanolreihe entwässern und lufttrocknen <p>HYDRATIERUNG GSPROZEDUR</p> <ol style="list-style-type: none"> Tropfen oder 20 µL der Sonde auf jedes Präparat geben und mit einem Deckglas abdecken Denaturieren Hydratisieren Entfernen der Deckgläser durch Einweichen in TBS Puffer 5-6 Tropfen PanWash (Weiß) zu jedem Präparat geben (mit Ausnahme der Positivkontrolle) Präparate in TBS Puffer spülen <p>DETEKTION UND FÄRBEPROCEDURE</p> <ol style="list-style-type: none"> 2-3 Tropfen konjugiert (Rot) auf jedes Präparat geben Präparate in TBS Puffer spülen Präparate mit destilliert/deionisiertem Wasser spülen Präparate mit destilliert/deionisiertem Wasser spülen Schema vorbereiten; AEC (blau) Gebrauchslösung nach nachfolgendem Schema vorbereiten: <table border="1"> <tr> <td>Multiplex Specimens</td> <td>Multiplex AEC Substrat</td> <td>Vol. AEC Buffer</td> </tr> <tr> <td>1-13</td> <td>4</td> <td>2 mL</td> </tr> <tr> <td>14-26</td> <td>8</td> <td>4 mL</td> </tr> <tr> <td>27-39</td> <td>12</td> <td>6 mL</td> </tr> <tr> <td>40-52</td> <td>16</td> <td>8 mL</td> </tr> </table> <p>Im dunkeln inkludieren</p> <ol style="list-style-type: none"> In destilliert/deionisiertem Wasser spülen Optional: 2-3 Tropfen der Gegenfärbung (Orange) auf jedes Präparat geben In destilliert/deionisiertem Wasser spülen Präparate für die mikroskopische Beurteilung abdecken 	Multiplex Specimens	Multiplex AEC Substrat	Vol. AEC Buffer	1-13	4	2 mL	14-26	8	4 mL	27-39	12	6 mL	40-52	16	8 mL	<p>2-16 Stunden bei 56-60°C</p> <p>2 x 10 Min.</p> <p>5 Min.</p> <p>30 Min. bei 37°C Heizplatte</p> <p>10 Min. bei 37°C Heizplatte</p> <p>10 Min. bei 37°C Heizplatte</p> <p>3 x 1 Min.</p> <p>5 Min. bei 95°C Heizplatte</p> <p>16 Stunden bei 37°C Ofen</p> <p>10 Min.</p> <p>15 Min. bei 37°C Heizplatte</p> <p>3 x 1 Min.</p> <p>30 Min. bei 37°C Heizplatte</p> <p>3 x 1 Min.</p> <p>1 Min.</p>
Multiplex Specimens	Multiplex AEC substrate	Vol. of AEC buffer																																
1-13	4	2 mL																																
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1-13	4	2 mL																																
14-26	8	4 mL																																
27-39	12	6 mL																																
40-52	16	8 mL																																



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.



Chapter 2 REMBRANDT® Universal DISH & HRP 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.

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. → 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 ± 5°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 ± 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 ₂ O ₂ /H ₂ 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.

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.

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 HRP-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.

Prepare during the last soak the AEC work solution in a disposable polypropylene tube or suitable glassware by mixing the AEC substrate with the AEC buffer (both blue) according the volumes given below. Do not make more work solution than necessary as it deteriorates within 3 hours after production. Keep the AEC work solution well protected from the light.

# specimens	# drops of AEC substrate	volume of AEC buffer
1-13	4	2 mL
14-26	8	4 mL
27-39	12	6 mL
40-52	16	8 mL

Take the slides out, wipe off excess of water and dry around the edges using a lint-free cloth. Ensure that the specimen on the slide is not disrupted. Apply 2-3 drops of AEC substrate (blue) to each specimen and transfer the slides onto a 37°C heating block or slide warmer. Incubate in the dark for 5-15 minutes at 37°C (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 Methyl Green (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 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.

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 complementary 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).

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 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.

- 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:

	<u>Positive Control DNA</u>	<u>Negative Control DNA</u>
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 Gynecology 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 β -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 Papilloma 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