

REMBRANDT®

In situ Hybridisation and Detection

DISH & AP Detection Kit-v5

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<i>DISH-AP kit for the detection of</i>	Biotin Label product code	Digoxigenin label product code	# Assays
HPV screening	A100K.0105	A100K.9905	40
HPV typing	A103K.0105	A103K.9905	40
CMV virus	A200K.0105	A200K.9905	40
Epstein-Barr virus	A300K.0105	A300K.9905	40
Herpes simplex virus	A400K.0105	A400K.9905	40

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- HYBRIDISATIE PROCEDURE**
1. Incubeer elk preparaat met probe reagens: 1 druppel of 20 µl endek af met de gelaaste
 2. Denatureer
 3. Verduif de fidegelaaste door preparaten in TBS buffer te dompelen
 4. Incubeer elk preparaat met PanWash (wit): 5-6 druppels
 5. met uitwisseling van de positieve controle
 6. Spoel alle preparaten in TBS buffer
- DETECTIE EN TEGENKLEURINGSPROCEDURE**
1. Incubeer elk preparaat met ontglat (rood): 2,3 druppels
 2. Spoel de preparaten met TBS buffer
 3. Spoel de preparaten met gelaaste reagenten/water
 4. Incubeer elk preparaat met NBT/BCIP substrant (blauw): 2,3 druppels en incubeer in donker
 5. Spoel de preparaten met gelaaste reagenten/water
 6. **Counterstain:** Incubeer elk preparaat met tegenkleuring (oranje): 2,3 druppels
 7. Spoel de preparaten met gelaaste reagenten/water
 8. Dekdecompes af

1. 5 min. bij 95°C hiele plaat
2. 16 uur bij 56-60°C
3. 10 min.
4. 15 min. bij 37°C hiele plaat
5. 3 x 1 min.
6. 30 min. bij 37°C hiele plaat
7. 3 x 1 min.
8. 5-15 min. bij 37°C hiele plaat
9. 3 x 1 min.
10. 1 min.
11. 3 x 1 min.

PROBE ²
DIGEST	PEPSIN POW
DIGEST	PEPSIN DIL
PROBE ²
CONTROL	+ ² DISH
CONTROL	- ² DISH
PANWASH	GC%.....50%
CONJ ² AP
SUBS	NBT/BCIP
WASH	TBS
COUNT	NFR

- PROCEDIMENTO DI IRRADIAZIONE**
1. Aggiungere 1 goccia o 20 µl di soluzione "probe" su ogni sezione.
 2. Coprire con coprivelino.
 3. Denaturare.
 4. Togliere il coprivelino scaldando il vetrino in tampone TBS.
 5. Aggiungere 5-6 goccie di PanWash (bianco) su ogni sezione.
 6. Lavare tutti i vetrini in tampone TBS.
- PROCEDIMENTO DI DETEZIONE E COLORAZIONE**
1. Aggiungere ad ogni sezione 2,3 goccie 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 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. **Counterstain:** aggiungere ad ogni sezione 2,3 goccie di "counterstain" (arancione).
 7. Lavare le sezioni in acqua distillata/deionizzata.
 8. Preparare le sezioni per l'osservazione al microscopio.

1. 5 min. subbocco riscaldante a 95°C
2. 16 ore a 37°C in incubatrice
3. 10 min.
4. 15 min. subbocco riscaldante a 37°C
5. 3 x 1 min.
6. 30 min. subbocco riscaldante a 37°C
7. 3 x 1 min.
8. 5-15 min. subbocco riscaldante a 37°C
9. 3 x 1 min.
10. 1 min.
11. 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 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® 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 ²	Yellow/Purple vial	:	Specific ¹ BIO or DIG labelled DNA probe(s)	0.8 mL
CONTROL	+ ² DISH	Pink vial	:	DISH positive control oligo probe (BIO or DIG)	0.8 mL
CONTROL	- ² DISH	Green vial	:	DISH negative control DNA probe(BIO or DIG)	0.8 mL
PANWASH	GC%.....50%	White vial	:	PanWash (differentiation reagent)	2 x 15 mL
CONJ ² 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

HANDLEIDING v4 DISH-AP

- SNIJDEN EN PLUKKEN VAN PARAFFINE COUPES**
1. Snij 4-6 µm coupes en plak ze op voorbehandelde objectglaasjes
 2. Verwarm de glasjes
 3. Deparaffineer in verse xyol
 4. Spoel in 100% ethanol en haal de glasjes lichtdrogen

- INCUBATIE TIJDEN**
1. 2-16 uur bij 56-60°C
 2. 2 x 10 min.
 3. 5 min.

VIAL LABEL

- PROTOCOLISCHE VOORBEREIDING**
1. Los het pepsine poeder (zoven) in 4 ml. gedestilleerd/gedeïoniseerd water op, verdeel in 150 µL porties en bewaar bij 20°C.
 2. Verdun het 1N HCl opspreef (transparant) tot de concentratie die voor de applicatie nodig is (oranje, cytologie of vries coupe zie 3)
 3. Verdun de proteolytische stock oplossing in het verdunde HCl en incubeer elk preparaat met 500-400 µL.

- INCUBATIE TIJDEN**
1. 30 min. bij 37°C hiele plaat
 2. 10 min. bij 37°C hiele plaat
 3. 10 min. bij 37°C hiele plaat
 4. 10 min. bij 37°C hiele plaat
 5. 3 x 1 min.

METHODICA D' USO v4 DISH-AP

- 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. Sdarcquare i vetrini in etanolo 100% ed asciugare all'aria

- TEMPI DI INCUBAZIONE**
1. 2-16 ore a 56-60°C
 2. 2 x 10 min.
 3. 5 min.



~ 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
CONTROL ²	White box C	: Positive control slides	2 pcs

¹ For specific probe specifications see chapter 5

² 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.



PREPARATION/USO DE LOS REAGENTES	PREPARAZIONE DEI REAGENTI
1. Preparar los serenos de 4 µm et coliar los en lames talladas	1. Preparare le sezioni di 4 µm et collare le su lame tagliate
2. Charlar las lames	2. Charlar le lame
3. Desparafinar dans du xylene frais	3. Desparaffinare in xileno fresco
4. Rembranz dans de l'ethanol 100% (ou ethanol absolu) et blissez sécher à l'air	4. Smergere le porte in etanol assoluto y seccare al aire

TEMPERATURE/INCUBAZIONE	TEMPERATURE DI INCUBAZIONE
2-16 hrs a 56-60°C	2-16 hrs a 56-60°C
2 x 10 min.	2 x 10 min.
5 min.	5 min.

PROTOCOLLO DI DETECCIONE E/DI CARICAZIONE	PROTOCOLLO DI RILEVAMENTO E CARICAZIONE
1. Distribuir 2-3 gotitas de control (rojo) per specimen	1. Distribuire 2-3 gotte di controllo (rosso) per specimen
2. Immerger las lames dans le tampon TBS	2. Immergere le lame dans le tampon TBS
3. Immerger las lames dans de l'eau distillée ou déionisée	3. Immergere le lame dans de l'eau distillée ou déionisée
4. Ajouter 2-3 gotites de PanWash (bleu) à chaque échantillon	4. Aggiungere 2-3 gocce di PanWash (blau) a chaque échantillon
5. Rinçer les lames avec de l'eau distillée ou déionisée	5. Rinçer le lame avec de l'eau distillée ou déionisée
6. Qualitatif ajouter 2-3 gotites de la solution colorante (orange)	6. Qualitativa aggiungere 2-3 gocce di la soluzione colorante (arancio)
7. Rinçer les lames avec de l'eau distillée ou déionisée	7. Rinçer le lame avec de l'eau distillée ou déionisée
8. Montrez les sections et examinez les lames au microscope	8. Montre le sezioni et examine le lame au microscope



PREPARATION OF PARAFFIN SECTIONS	INCUBATION TIME	PROBE
1. Cut 46 µm sections and collect on treated glass slides	2-16 hrs at 56-60°C	
2. Heat slides	2 x 10 min.	
3. Dewax in fresh xylene	5 min.	
4. Soak slides in 100% ethanol and air dry		
PROTEOLYTIC TREATMENT		
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		
2. Dilute the 1N HCl pepsin diluent (transparent) to the application required concentration (paraffin, cytological or frozen, see 3)		
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 Cytological: 25,000x in 0.01N HCl; add 4 µL to 100 mL 0.01N HCl; Frozen: 50,000x in 0.01N HCl; add 2 µL to 100 mL 0.01N HCl	30 min. 37°C heating block 10 min. 37°C heating block 10 min. 37°C heating block 3 x 1 min.	DIGEST Pepsin Pow DIL Pepsin Dil
HYDRIDIZATION PROCEDURE		
1. Apply 1 drop or 20 µL of probe solution per specimen; cover with coverslip		
2. Denature	5 min. 95°C heating block	
3. Hydrate	16 hrs 37°C incubator	
4. Remove coverslips by soaking slides in TBS buffer	10 min.	
5. Apply 5-6 drops of PanWash (white) to each specimen except the positive control	15 min. 37°C heating block	
6. Wash all slides in TBS buffer	3 x 1 min.	
DETECTION AND STAINING PROCEDURE		
1. Apply 2-3 drops of the conjugate (red) to each specimen	30 min. 37°C heating block	
2. Soak slides in TBS buffer	3 x 1 min.	
3. Soak slides in destaining/deionized water	1 min.	
4. Apply 2-3 drops of NB1/BCP substrate (blue) to each specimen and include in dark	5-15 min. 37°C heating block	
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.	
7. Wash slides in distilled/deionized water	3 x 1 min.	
8. Mount sections for microscopic evaluation		

REFERENCE GUIDE-V4 DISH-AP

VIAL-LABEL

HERSTELLUNG VON PARAFFINSEKTIONEN	INKUBATIONSZEITEN	PROBE
1. 46 µm Schnitte anfertigen und auf vorbereitete Objektträger ziehen	2-16 Stunden bei 56-60°C	
2. Schnitte in Xylenen einweichen	2 x 10 Min.	
3. in Xylenen entfärben	5 Min.	
4. Schnitte in 100% Ethanol einwaschen und lufttrocknen		
PROTEOLYTISCHE BEHANDLUNG		
1. Pepsin-Pulver (Schwarz) in 4 mL destilliertem/entionisiertem 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 (Paraffin: 100x, Zytologisches Präparat oder Gefrierschnitt: siehe 3)		
3. Verdünne die aufgetaute proteolytische Lösung in verdünntem HCl; jedes Präparat mit 300-400 µL inkubieren. Paraffin: 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 Gefrierschnitt: 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 3 x 1 Min.	DIGEST Pepsin Pow DIL Pepsin Dil
HYDRIDISIERUNGSPROZEDUR		
1. 1 Tropfen oder 20 µL der Sonde auf jedes Präparat geben und mit einem Deckglas abdecken		
2. Denaturieren	5 Min. bei 95°C Heizplatte	
3. Hydratisieren	16 Stunden bei 37°C Ofen	
4. Entfernen der Deckgläser durch Einweichen in TBS-Puffer	10 Min.	
5. 5-6 Tropfen PanWash (Weiß) zu jedem Präparat geben (mit Ausnahme der Positivkontrolle)	15 Min. bei 37°C Heizplatte	
6. 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/entionisiertem Wasser spülen	1 Min.	
4. 2-3 Tropfen des NB1/BCP-Substrats (Blau) auf jedes Präparat geben im dunklen Inkubator	5-15 Min. bei 37°C Heizplatte	
5. überschüssigen Substrat-Wasser spülen	3 x 1 Min.	
6. Optional: 2-3 Tropfen der Gegenfärbelösung (Orange) auf jedes Präparat geben	1 Min.	
7. indistilliertem/entionisiertem Wasser spülen	3 x 1 Min.	
8. Präparate für die mikroskopische Beurteilung abdecken		

ANLEITUNG-V4 DISH-AP



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® 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 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 probe solution (yellow/purple) 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.

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.

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

- 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 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:

	HPV ¹	CMV	HSV	EBV	Positive Control DNA	Negative Control DNA
Specificity	95%	100%	100%	100%	100%	100%
Sensitivity	85%	85%	85%	85%	95%	95%

¹ The specificity of the HPV DNA probes is 100% for the HPV species, but the different specific HPV probe sub-types may show some inter-type cross reactions.

Chapter 4 References

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Chapter 5 Probe specifications

REMBRANDT® Biotin¹ and Digoxigenin² labelled DNA probe specifications

PRODUCT CODE	LABEL	DNA PROBE SPECIFICATIONS			
		Description	Size	Region	Vector
A100P.0100 A100P.9900	BIO DIG	Human papilloma virus HPV screening DNA probe (PROBE xxx panHPV)*	100-300 bp	mix of total genomes 7-8 Kb: containing the conserved HPV region	pBR322: 4.3 Kb and pSP; 3.0 Kb
A191P.0100 A191P.9900	BIO DIG	Human papilloma virus type 6/11 DNA probe (PROBE xxx HPV 6/11)*	100-300 bp	total genome 7.8 Kb HPV type 6 and 7.9 Kb HPV type 11	pSP; 3.0 Kb
A192P.0100 A192P.9900	BIO DIG	Human papilloma virus type 16/18 DNA probe (PROBE xxx HPV 16/18)*	100-300 bp	total genome 7.9 Kb HPV type 16 and 7.9 Kb HPV type 18	pSP; 3.0 Kb
A193P.0100 A193P.9900	BIO DIG	Human papilloma virus type 31/33 DNA probe (PROBE xxx HPV 31/33)*	100-300 bp	total genome 7.9 Kb HPV type 31 and 7.9 Kb HPV type 33	pBR322: 4.3 Kb and modified pSP ≈ 4.0 Kb
A106P.0100 A106P.9900	BIO DIG	Human papilloma virus type 6 DNA probe (PROBE xxx HPV 6)*	100-300 bp	total genome 7.8 Kb HPV type 6	pSP; 3.0 Kb
A111P.0100 A111P.9900	BIO DIG	Human papilloma virus type 11 DNA probe (PROBE xxx HPV 11)*	100-300 bp	total genome 7.9 Kb HPV type 11	pSP; 3.0 Kb
A116P.0100 A116P.9900	BIO DIG	Human papilloma virus type 16 DNA probe (PROBE xxx HPV 16)*	100-300 bp	total genome 7.9 Kb HPV type 16	pSP; 3.0 Kb
A118P.0100 A118P.9900	BIO DIG	Human papilloma virus type 18 DNA probe (PROBE xxx HPV 18)*		total genome 7.9 Kb HPV type 18	pSP; 3.0 Kb
A131P.0100 A131P.9900	BIO DIG	Human papilloma virus type 31 DNA probe (PROBE xxx HPV 31)*	100-300 bp	total genome 7.9 Kb HPV type 31	pBR322: 4.3 Kb
A133P.0100 A133P.9900	BIO DIG	Human papilloma virus type 33 DNA probe (PROBE xxx HPV 33)*	100-300 bp	total genome 7.9 Kb HPV type 33	modified pSP ≈ 4.0 Kb
A200P.0100 A200P.9900	BIO DIG	Human Cytomegalovirus DNA probe (PROBE xxx CMV)*	100-300 bp	total genome 235 Kb	
A300P.0100 A300P.9900	BIO DIG	Epstein-Barr virus DNA probe (PROBE xxx EBV)*	100-300 bp	W fragment: 4.2 Kb	pDR720: 4.0 Kb
A400P.0100 ** A400P.9900 **	BIO DIG	Herpes simple x virus type 1/2 DNA probe (PROBE xxx HSV 1/2)*	100-300 bp	pSPM: 2.6 Kb	three HSV2 Sma I fragments: total=3.0Kb
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)

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

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).