DATA SHEET-V1
REMBRANDT® KALLMANN (KAL1) SYNDROME FISH DETECTION

CE IVD

PanPath

Ref		
C803K.2030.05	Σ	5 T
C803K.2030.10	Σ	10 T

Intended use

I. The REMBRANDT® Kallmann (KAL1) syndrome FISH assay is an *in-vitro* diagnostics medical device intended for the detection of a deletion in the Kallmann syndrome critical region Xp22.31, in comparison to the centromeric region of the human chromosome X, by means of *in situ* hybridization.

II. The REMBRANDT® Kallmann (KAL1) syndrome FISH assay is intended for the detection of a deletion in the Kallmann syndrome critical region Xp22.31, in comparison to the centromeric region of the human chromosome X in fixed cells. 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 other possible test data.

III. The REMBRANDT® Kallmann (KAL1) syndrome FISH assay kit is a qualitative assay for the detection of a deletion in the Kallmann syndrome critical region Xp22.31, in comparison to the centromeric region of the human chromosome X.

IV. The intended users are qualified laboratory employees in cytology and/or pathology. The product is intended for professional use.

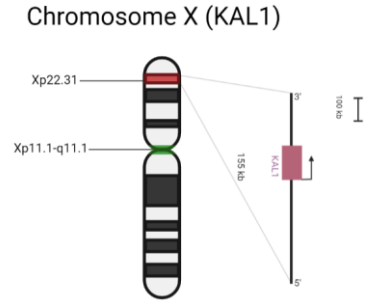
Clinical relevance

The Xp22.31 microdeletion syndrome, also known as the X-linked Kallmann syndrome KAL1. In female, the syndrome is caused by a heterozygous deletion of Xp22.31. In male, the syndrome is caused by a hemizygous deletion of Xp22.31, due to the lack of 2 X-chromosomes. The Kallmann syndrome has a prevalence in female of about 1 in 40,000 and in male of about 1 in 8,000 births worldwide (Dodé & Hardelin, 2009). KAL1 is characterized by the inability to start puberty, hypogonadism and bimanual synkinesis, in which the movements of one hand are mirrored by the other hand (Balasubramanian & Crowley, 1993). The KAL1 (Kallmann syndrome 1 sequence) gene, also known as the ANOS1 (anosmin 1) gene, is responsible for the X-linked form of KS (AbuJbara et al., 2004).

The ANOS1 gene has a pseudogene on the Y chromosome called ANOS2P, which is a nonfunctional gene that resembles the functional ANOS1 gene (de Castro et al., 2017; Dodé & Hardelin, 2009). REMBRANDT® KAL1 FISH probes are designed to target the KAL1 critical region, mapping the ANOS1 gene of 155 kb long.

Probe specification

The REMBRANDT® Kallmann probe set consists of a 155 kb probe detecting the Xp22.31 locus and a 0.68 kb centromeric probe detecting the centromeric region of chromosome X. The centromeric region is detected by green fluorescence (AF488) and the locus is detected by orange fluorescent detection (AF555). The Kallmann (KAL1) syndrome FISH assay is able to detect a deletion in the Kallmann syndrome critical region Xp22.31, in comparison to the centromeric region of the human chromosome X by means of direct *in situ* hybridization. The Kallmann probes are pre-mixed in a hybridization mixture (formamide, dextran sulphate and SSC) and are ready to use solutions.



Test principle

In a fluorescent *in situ* hybridization assay, a double stranded DNA probe labelled with a fluorochrome is used. The labelled DNA probe is diluted in a hybridization mixture. The hybridization mixture containing the DNA probe is added to the specimen. A co-denaturation will ensure that the genomic DNA and the probe DNA become single stranded. After denaturation, the probe DNA is able to hybridize to its complementary target sequences in the cells. In the Rembrandt® Kallmann (KAL1) syndrome FISH assay, the fluorochrome is attached to the probe and the signals can be visualized directly by fluorescent microscopy after hybridization.

Reagents provided

Product name	Product number	Amount
Labelled probe (depending on label and size choice)		
●KS -FISH probes orange/ green detection	C803P.2030.05	Σ 5 T
●KS -FISH probes orange/ green detection	C803P.2030.10	Σ 10 T
Pepsin powder	R011R.0000	1 g
Pepsin diluent	R018R.0000	15 ml
PanWash 4, 25X SSC	R025R.0000	15 ml
Fluorescent Mounting medium	Z000R.0050	1 ml

Assay procedure

REMBRANDT® Kallmann (KAL1) syndrome FISH detection assay procedure for cytological specimen.

I. Incubate slides in pre-heated proteolytic work solution (prepare according to section 1.9 of Manual-FISH) (R011R.000 + R018R.000) at 37 ˚C (100 µg/ml) for 15 minutes followed by a brief rinsing in 0.01M HCl (1x 2 minutes) and subsequent rinses in PBS (2x 1 minute)

II. Flush wash slides in deionised water, followed by dehydration in graded ethanol series (ethanol 70%, 96%, 96%, 100%, 100%) 1 minute each and air-dry slides for 15 minutes

Do not treat more than 5 slides at the same time, because the temperature of the pre-heated solutions may drop dramatically, thus causing incomplete pre-treatment. Additionally, allow the slides to air-dry as recommended; otherwise sections will be lost.

III. Homogenize probe solution (C803P.2030.YY) a spin briefly. Apply 15 µl of probe solution to each specimen. Cover all specimens with a cover slip (avoid air bubbles). Denature slides on a 80 ˚C hotplate or other heating device for 3 minutes

Work in a pre-set order to ensure that all slides have been incubated at 80 ˚C for the exact same time. Do not denature more than 5 slides at the same time, the temperature of the heating device may drop dramatically, thus causing incomplete denaturation.

IV. Transfer the slides into a moist environment and incubate for 16 hours at 37 ˚C.

V. Remove coverslips by soaking the slides in PBS at room temperature

VI. Incubate the slides in diluted, pre-heated PanWash 4 (R025R.000) (prepare according to section 1.9 of Manual-FISH)

Do not incubate more than 5 slides at the same time in PanWash 4, the temperature of the PanWash 4 may drop dramatically, causing wrong stringency conditions.

VII. Incubate the slides in PBS at room temperature for 1 minute

VIII. Dehydrate the slides in graded ethanol series (70%, 96%, 96%, 100%, 100%) 1 minute each and air-dry the slides for 15 minutes (in the dark)

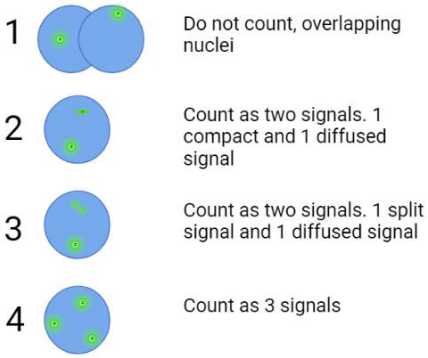
IX. Mount the slides by applying mounting medium (Z000R.0050) and coverslip

Interpretation of results

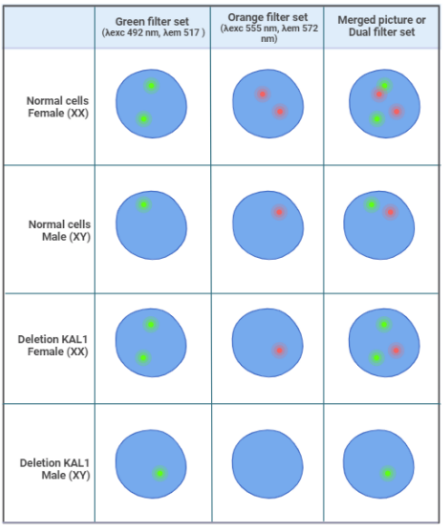
Hybridization of the Kallmann probes is viewed using a fluorescence microscope equipped with appropriate excitation and emission filters for orange detection: λ_{exc} 555 nm, λ_{em} 572 nm. Allowing visualization of orange fluorescent signal concentrated at the Xp22.31 locus of chromosome X in combination with green fluorescent signals representing the centromeric region of chromosome X. The enumeration Xp22.31 copy numbers conducted by microscopic examination of interphase nuclei, compared to the signals representing chromosome X. The fluorescently-stained locus of chromosome X stand out brightly against the general fluorescence of the nucleus. The Kallmann (KAL1) syndrome FISH procedure enables observation of a possible detection of a deletion in the Kallmann syndrome critical region Xp22.31, in comparison to the centromeric region of the human chromosome X within the nuclei. The assay results are reported as the percentage of nuclei with 0, 1, 2, 3, 4, and >4 fluorescent signals. Each orange fluorescent signal corresponds to the Xp22.31 locus, and each green fluorescent signal corresponds to the centromere of chromosome X.
Enumerate the fluorescent signals in the interphase nucleus using a 40X or 63X magnification. Objectives with higher magnification (eg, 63X or 100X) should be used to verify or resolve questions about split or diffused signals. Enumerate at least 100 nuclei per slide for accurate analysis.

•Two signals in close proximity and approximately the same sizes but not connected by a visible link are counted as 2 signals.
•Count a diffuse signal as 1 signal if diffusion of the signal is contiguous and within an acceptable boundary.
•Two small signals connected by a visible link are counted as 1 signal.
•Enumerate the number of nuclei with 0, 1, 2, 3, 4, or >4 signals. Count nuclei with 0 signals only if there are other nuclei with at least 1 signal present in the field of view. If

the accuracy of the enumeration is in doubt, repeat the enumeration in another area of the slide.
•Do not enumerate nuclei with uncertain signals (’Marilyn S., 2017)



Expected results in normal and aberrant nuclei



Other signal distribution may be observed in some abnormal samples which might result in a different signal pattern than described above. Unexpected signal patterns should be further investigated.

Performance characteristics

Analytical Sensitivity and Specificity

The analytical sensitivity and specificity were investigated within PanPaths analytical performance assessment. Precision was investigated for the Kallmann (KAL1) syndrome FISH assay and results are available upon request.

Analytical sensitivity

The analytical sensitivity was determined in three levels. The normal cut-off percentage was determined based on assessment of 200 individual nuclei in two samples of healthy interphase lymphocytes from peripheral blood. The beta inversion was used to determine the percentage of normal-cut off. For the noise-to-signal percentage, the noise and signal values were determined for 100 signals in interphase lymphocytes from peripheral blood of two independent samples. The differences between noise and signal were evaluated using a 95% confidence interval. For the hybridization efficiency, 200 individual nuclei were assessed for the presence of FISH signals.

Performance characteristic	Outcome
Normal cut-off percentage	8.6%
Noise-to-signal cut-off percentage	32%
Hybridization efficiency	99%

Analytical specificity

The analytical specificity was determined in two levels. The theoretical specificity was determined by sequencing analysis of the probe DNA and mapping on Hg38. The practical specificity was determined by assessing the hybridization pattern in metaphase chromosomes of interphase lymphocytes from peripheral blood.

Performance characteristic	Outcome
Theoretical specificity	Mapped on chromosome the locus Xp22.31 and the centromeric region Xp11.1-q11.1
Practical specificity	100%

Limitations of Procedure

i) The REMBRANDT® Kallmann (KAL1) syndrome FISH assay is solely applicable detection of a deletion in the Kallmann syndrome critical region Xp22.31, in comparison to the centromeric region of the human chromosome X, which may be present in cell preparations (cytological specimen i.e. interphase lymphocytes from peripheral blood samples).

ii) Either human tissue sections or human cytological preparations may be used. Samples must be fixed in buffered formalin or alcohol. In tissue sections are required, the sections should be prepared in a 4 µm thickness. Furthermore, the tissues should be glued to the glass slides with a bio-adhesive (e.g. organ silane), dried at room temperature, subsequently dried at 37 °C overnight and lastly completely deparaffinized in xylene and alcohol series and air dried.

iii) 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.

iv) 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.

v) The performance of the ISH procedure is also affected by the sensitivity of the method and the presence of the centromeric region of chromosome. In case the limit of the sensitivity is reached a false negative reaction may be the result.

vi) The REMBRANDT® Kallmann (KAL1) syndrome FISH assay results should not be relied on in case the sampling, sampling method, sample quality, sample preparation, reagents used, controls and procedure followed are not optimal or as described the working protocols.

vii) The clinical interpretation of the results should not be established on the basis of a single test result. A precise diagnosis should take into consideration clinical history as well as data obtained from other molecular test (i.e. WGS).

viii) Therapeutic considerations based on the result of this test alone should not been taken. Results should be verified by other traditional diagnostic methods such as but not limited to clinical history, symptoms, as well as morphological data.

ix) The medical profession should be aware of risks and factors influencing the fluorescent signal intensity while interpretating the test result. Microscopy settings might influence the signal intensity and/or interpretation.

x) Laboratory personnel performing the test should be trained and knowledgeable to be able to interpret the test results.

Storage and handling

Store kit and its contents at 2-8°C. Store the dissolved and aliquoted reagents at recommended temperatures. When used and stored as indicated, the kit is stable until the expiry date printed on the box.

Product	Product number	Storage conditions
Xp22.31 CEPX Probe orange /green detection	C803P.2030	2-8 ˚C
Pepsin powder	R011R.0000	Powder: 2-25˚C, ambient temperature Dissolved: -20˚C
Pepsin diluent	R018R.0000	Concentrated solution and diluted: 2-25˚C, ambient temperature
PanWash 4, 25X SSC	R025R.0000	Concentrated solution and diluted: 2-25˚C, ambient temperature
Fluorescent mounting medium	Z000R.0050	2-8 ˚C

Hazard statements

H315 - Causes skin irritation
H319 - Causes serious eye irritation
H351 - Suspected of causing cancer
H360D - May damage the unborn child
H373 - May cause damage to organs through prolonged or repeated exposure

Precautionary Statements

P202 - Do not handle until all safety precautions have been read and understood
P280 - Wear protective gloves/protective clothing/eye protection/face protection
P302 + P352 - IF ON SKIN: Wash with plenty of water and soap
P305 + P351 + P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
P308 + P313 - IF exposed or concerned: Get medical advice/attention
P362 + P364 - Take off contaminated clothing and wash it before reuse
P405 - Store locked up

Additional information

Product in combination with other devices

The REMBRANDT® DNA probes are intended for stand-alone usage. The in vitro diagnostic is intended to be used in combination with standard cytological preparation methods, hot plate(s), stove(s), incubation device(s), water bath(s), temperature and incubation time control(s), proteolytic-, detection- and other reagents (not supplied with this reagent) and a microscope. The combination has been tested and validated. Since the 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 proteolytic reagents, detection 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 clinical state of the sample, which may influence the validation criteria.

For additional information regarding the Rembrandt® assays, a manual is included which specifies the following subjects:

- Controls
- Materials required but not included
- Storage and shelf-life

- Performance precautions
- Preparations of reagents
- Specimen collection
- Quality control
- Trouble shooting guide

Technical assistance

For technical assistance regarding the products performance, please contact info@panpath.nl or call +31 495499090. Visit our website for reprints of datasheets or additional documentation. www.panpath.nl

Literature list

AbuJbara, M. A., Hamamy, H. A., Jarrah, N. S., Shegem, N. S., & Ajlouni, K. M. (2004). Clinical and inheritance profiles of Kallmann syndrome in Jordan. *Reproductive Health*, *1*(1), 5. https://doi.org/10.1186/1742-4755-1-5

Balasubramanian, R., & Crowley, W. F. (1993). *Isolated Gonadotropin-Releasing Hormone (GnRH) Deficiency*.

de Castro, F., Seal, R., & Maggi, R. (2017). ANOS1: a unified nomenclature for Kallmann syndrome 1 gene (KAL1) and anosmin-1. *Briefings in Functional Genomics*, *16*(4), 205–210. https://doi.org/10.1093/bfgp/elw037

Dodé, C., & Hardelin, J.-P. (2009). Kallmann syndrome. *European Journal of Human Genetics*, *17*(2), 139–146. https://doi.org/10.1038/ejhg.2008.206

'Marilyn S., A. 'Margaret J. , B. ' H. J. , L. (2017). *The AGT Cytogenetics Laboratory Manual* (4th Edition).

DATA SHEET-V1
REMBRANDT® LSI XP22.31/CEP X-FISH DETECTION
RESEARCH USE ONLY (RUO)

RUO

PanPath

Ref

C803K.2030.05	Σ	5 T
C803K.2030.10	Σ	10 T

Intended use

I. The REMBRANDT® LSI Xp22.31/CEP X-FISH assay is for research use only and is intended for the detection of the locus Xp22.31, in comparison to the centromeric region of the human chromosome X in fixed cells.

II. The REMBRANDT® LSI Xp22.31/CEP X-FISH assay kit is a qualitative assay for the detection of the locus Xp22.31, in comparison to the centromeric region of the human chromosome X. A clinical diagnosis should not be established based on the performance of this test.

III. The intended users are qualified laboratory employees in cytology and/or pathology. The product is intended for professional use.

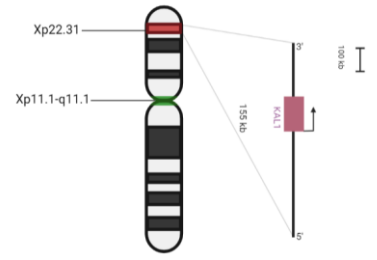
Clinical relevance

The Xp22.31 microdeletion syndrome, also known as the X-linked Kallmann syndrome KAL1. In female, the syndrome is caused by a heterozygous deletion of Xp22.31. In male, the syndrome is caused by a hemizygous deletion of Xp22.31, due to the lack of 2 X-chromosomes. The Kallmann syndrome has a prevalence in female of about 1 in 40,000 and in male of about 1 in 8,000 births worldwide (Dodé & Hardelin, 2009). KAL1 is characterized by the inability to start puberty, hypogonadism and bimanual synkinesis, in which the movements of one hand are mirrored by the other hand (Balasubramanian & Crowley, 1993). The KAL1 (Kallmann syndrome 1 sequence) gene, also known as the ANOS1 (anosmin 1) gene, is responsible for the X-linked form of KS (AbuJbara et al., 2004).
The ANOS1 gene has a pseudogene on the Y chromosome called ANOS2P, which is a nonfunctional gene that resembles the functional ANOS1 gene (de Castro et al., 2017; Dodé & Hardelin, 2009) .Rembrandt KAL1 FISH probes are designed to target the KAL1 critical region, mapping the ANOS1 gene of 155 kb long.

Probe specification

The REMBRANDT® Xp22.31/CEP X probe set consists of a 155 kb probe detecting the Xp22.31 locus and a 0.68 kb centromeric probe detecting the centromeric region of chromosome X.

The centromeric region is detected by green fluorescence (AF488) and the locus is detected by orange fluorescent detection (AF555). The Kallmann (KAL1) syndrome FISH assay is able to detect a deletion in the Kallmann syndrome critical region Xp22.31, in comparison to the centromeric region of the human chromosome X by means of direct *in situ* hybridization. The Kallmann probes are pre-mixed in a hybridization mixture (formamide, dextran sulphate and SSC) and are ready to use solutions.



Test principle

In a fluorescent *in situ* hybridization assay, a double stranded DNA probe labelled with a fluorochrome is used. The labelled DNA probe is diluted in a hybridization mixture. The hybridization mixture containing the DNA probe is added to the specimen. A co-denaturation will ensure that the genomic DNA and the probe DNA become single stranded. After denaturation, the probe DNA is able to hybridize to its complementary target sequences in the cells. In the REMBRANDT® LSI Xp22.31/CEP X-FISH assay, the fluorochrome is attached to the probe and the signals can be visualized directly by fluorescent microscopy after hybridization.

Reagents provided

Product name	Product number	Amount
Labelled probes (depending on label and size choice)		
●LSI Xp22.31/CEP X -FISH probes orange/green detection	C803P.2030.05	Σ 5 T
●LSI Xp22.31/CEP X -FISH probes orange/green detection	C803P.2030.10	Σ 10 T
Pepsin powder	R011R.0000	1 g
Pepsin diluent	R018R.0000	15 ml
PanWash 4, 25X SSC	R025R.0000	15 ml

Fluorescent Mounting medium	Z000R.0050	1 ml

Assay procedure

REMBRANDT® LSI Xp22.31/CEP X-FISH detection assay procedure for cytological specimen.

I. Incubate slides in pre-heated proteolytic work solution (prepare according to section 1.9 of Manual-FISH) (R011R.000 + R018R.000) at 37 ˚C (100 µg/ml) for 15 minutes followed by a brief rinsing in 0.01M HCl (1x 2 minutes) and subsequent rinses in PBS (2x 1 minute)

II. Flush wash slides in deionised water, followed by dehydration in graded ethanol series (ethanol 70%, 96%, 96%, 100%, 100%) 1 minute each and air-dry slides for 15 minutes

Do not treat more than 5 slides at the same time, because the temperature of the pre-heated solutions may drop dramatically, thus causing incomplete pre-treatment. Additionally, allow the slides to air-dry as recommended; otherwise sections will be lost.

III. Homogenize probe solution (C803P.2030.YY) a spin briefly. Apply 15 µl of probe solution to each specimen. Cover all specimens with a cover slip (avoid air bubbles). Denature slides on a 80 ˚C hotplate or other heating device for 3 minutes

Work in a pre-set order to ensure that all slides have been incubated at 80 ˚C for the exact same time. Do not denature more than 5 slides at the same time, the temperature of the heating device may drop dramatically, thus causing incomplete denaturation.

IV. Transfer the slides into a moist environment and incubate for 16 hours at 37 ˚C.

V. Remove coverslips by soaking the slides in PBS at room temperature

VI. Incubate the slides in diluted, pre-heated PanWash 4 (R025R.000) (prepare according to section 1.9 of Manual-FISH)

Do not incubate more than 5 slides at the same time in PanWash 4, the temperature of the PanWash 4 may drop dramatically, causing wrong stringency conditions.

VII. Incubate the slides in PBS at room temperature for 1 minute

VIII. Dehydrate the slides in graded ethanol series (70%, 96%, 96%, 100%, 100%) 1 minute each and air-dry the slides for 15 minutes (in the dark)

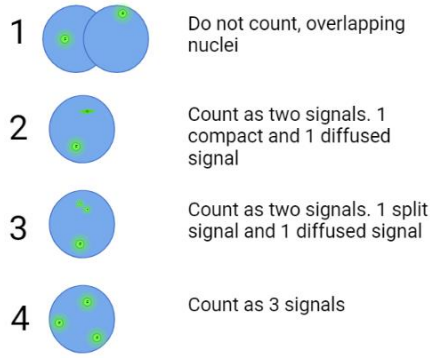
IX. Mount the slides by applying mounting medium (Z000R.0050) and coverslip

Interpretation of results

Hybridization of the LSI Xp22.31/CEP X probes is viewed using a fluorescence microscope equipped with appropriate excitation and emission filters for orange detection: λ_{exc} 555 nm, λ_{em} 572 nm. Allowing visualization of orange fluorescent signal concentrated at the Xp22.31 locus of chromosome X in combination with green fluorescent signals representing the centromeric region of chromosome X. The enumeration Xp22.31 copy numbers conducted by microscopic examination of interphase nuclei, compared to the signals representing chromosome X. The fluorescently-stained locus of chromosome X stand out brightly against the general fluorescence of the nucleus. The assay results are reported as the percentage of nuclei with 0, 1, 2, 3, 4, and >4 fluorescent signals. Each orange fluorescent signal corresponds to the Xp22.31 locus, and each green fluorescent signal corresponds to the centromere of chromosome X.

Enumerate the fluorescent signals in the interphase nucleus using a 40X or 63X magnification. Objectives with higher magnification (eg, 63X or 100X) should be used to verify or resolve questions about split or diffused signals. Enumerate at least 100 nuclei per slide for accurate analysis.

•Two signals in close proximity and approximately the same sizes but not connected by a visible link are counted as 2 signals.
•Count a diffuse signal as 1 signal if diffusion of the signal is contiguous and within an acceptable boundary.
•Two small signals connected by a visible link are counted as 1 signal.
•Enumerate the number of nuclei with 0, 1, 2, 3, 4, or >4 signals. Count nuclei with 0 signals only if there are other nuclei with at least 1 signal present in the field of view. If the accuracy of the enumeration is in doubt, repeat the enumeration in another area of the slide.
•Do not enumerate nuclei with uncertain signals (’Marilyn S., 2017)



Performance characteristics

Analytical Sensitivity and Specificity

The analytical sensitivity and specificity were investigated within PanPaths analytical performance assessment. Precision was investigated for the LSI Xp22.31/CEP X-FISH assay and results are available upon request.

Analytical sensitivity

The analytical sensitivity was determined in three levels. The normal cut-off percentage was determined based on assessment of 200 individual nuclei in two samples of healthy interphase lymphocytes from peripheral blood. The beta inversion was used to determine the percentage of normal-cut off. For the noise-to-signal percentage, the noise and signal values were determined for 100 signals in interphase lymphocytes from peripheral blood of two independent samples. The differences between noise and signal were evaluated using a 95% confidence interval. For the hybridization efficiency, 200 individual nuclei were assessed for the presence of FISH signals.

Performance characteristic	Outcome
Normal cut-off percentage	8.6%
Noise-to-signal cut-off percentage	32%
Hybridization efficiency	99%

Analytical specificity

The analytical specificity was determined in two levels. The theoretical specificity was determined by sequencing analysis of the probe DNA and mapping on Hg38. The practical specificity was determined by assessing the hybridization pattern in metaphase chromosomes of interphase lymphocytes from peripheral blood.

Performance characteristic	Outcome
Theoretical specificity	Mapped on chromosome the locus Xp22.31 and the centromeric region Xp11.1-q11.1
Practical specificity	100%

Limitations of Procedure

i) The LSI Xp22.31/CEP X FISH assay is solely applicable detection the Xp22.31 locus, in comparison to the centromeric region of the human chromosome X, which may be present in cell preparations (cytological specimen i.e. interphase lymphocytes from peripheral blood samples).

ii) Either human tissue sections or human cytological preparations may be used. Samples must be fixed in buffered formalin or alcohol. In tissue sections are required, the sections should be prepared in a 4 µm thickness. Furthermore, the tissues should be glued to the glass slides with a bio-adhesive (e.g. organ silane), dried at room temperature, subsequently dried at 37 °C overnight and lastly completely deparaffinized in xylene and alcohol series and air dried.

iii) 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.

iv) 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.

v) The performance of the ISH procedure is also affected by the sensitivity of the method and the presence of the Xp22.31 locus and the centromeric region of chromosome X. In case the limit of the sensitivity is reached a false negative reaction may be the result.

vi) The LSI Xp22.31/CEP X-ISH assay results should not be relied on in case the sampling, sampling method, sample quality, sample preparation, reagents used, controls and procedure followed are not optimal or as described the working protocols.

vii) The medical profession should be aware of risks and factors influencing the fluorescent signal intensity while interpretating the test result. Microscopy settings might influence the signal intensity and/or interpretation.

viii) Laboratory personnel performing the test should be trained and knowledgeable to be able to interpret the test results.

Storage and handling

Store kit and its contents at 2-8°C. Store the dissolved and aliquoted reagents at recommended temperatures. When used and stored as indicated, the kit is stable until the expiry date printed on the box.

Product	Product number	Storage conditions
Xp22.31/CEP X Probe orange /green detection	C803P.2030	2-8 ˚C
Pepsin powder	R011R.0000	Powder: 2-25˚C, ambient temperature Dissolved: -20˚C
Pepsin diluent	R018R.0000	Concentrated solution and diluted: 2-25˚C, ambient temperature
PanWash 4, 25X SSC	R025R.0000	Concentrated solution and diluted: 2-25˚C,

Fluorescent mounting medium	Z000R.0050	ambient temperature 2-8 ˚C

Hazard statements

H315 - Causes skin irritation
H319 - Causes serious eye irritation
H351 - Suspected of causing cancer
H360D - May damage the unborn child
H373 - May cause damage to organs through prolonged or repeated exposure

Precautionary Statements

P202 - Do not handle until all safety precautions have been read and understood
P280 - Wear protective gloves/protective clothing/eye protection/face protection
P302 + P352 - IF ON SKIN: Wash with plenty of water and soap
P305 + P351 + P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
P308 + P313 - IF exposed or concerned: Get medical advice/attention
P362 + P364 - Take off contaminated clothing and wash it before reuse
P405 - Store locked up

Additional information

Product in combination with other devices

The REMBRANDT® DNA probes are intended for stand-alone usage. The in vitro diagnostic is intended to be used in combination with standard cytological preparation methods, hot plate(s), stove(s), incubation device(s), water bath(s), temperature and incubation time control(s), proteolytic-, detection- and other reagents (not supplied with this reagent) and a microscope. The combination has been tested and validated. Since the 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 proteolytic reagents, detection 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 clinical state of the sample, which may influence the validation criteria.

For additional information regarding the Rembrandt® assays, a manual is included which specifies the following subjects:

- Controls
- Materials required but not included
- Storage and shelf-life
- Performance precautions
- Preparations of reagents
- Specimen collection
- Quality control
- Trouble shooting guide

Technical assistance

For technical assistance regarding the products performance, please contact info@panpath.nl or call +31 495499090. Visit our website for reprints of datasheets or additional documentation. www.panpath.nl

Literature list

AbuJbara, M. A., Hamamy, H. A., Jarrah, N. S., Shegem, N. S., & Ajlouni, K. M. (2004). Clinical and inheritance profiles of Kallmann syndrome in Jordan. *Reproductive Health*, *1*(1), 5. https://doi.org/10.1186/1742-4755-1-5

Balasubramanian, R., & Crowley, W. F. (1993). *Isolated Gonadotropin-Releasing Hormone (GnRH) Deficiency*.

de Castro, F., Seal, R., & Maggi, R. (2017). ANOS1: a unified nomenclature for Kallmann syndrome 1 gene (KAL1) and anosmin-1. *Briefings in Functional Genomics*, *16*(4), 205–210. https://doi.org/10.1093/bfgp/elw037

Dodé, C., & Hardelin, J.-P. (2009). Kallmann syndrome. *European Journal of Human Genetics*, *17*(2), 139–146. https://doi.org/10.1038/ejhg.2008.206

'Marilyn S., A. 'Margaret J. , B. ' H. J. , L. (2017). *The AGT Cytogenetics Laboratory Manual* (4th Edition).

DATA SHEET-V1
REMBRANDT® LSI XP22.31/CEP X-ISH DETECTION
RESEARCH USE ONLY (RUO)

RUO

PanPath

Ref			
	C803K.0199.05	Σ	5 T
	C803K.0199.10	Σ	10 T

Intended use

I. The REMBRANDT® LSI Xp22.31/CEP X- ISH detection assay is an in-vitro diagnostics medical device intended for the detection of the Xp22.31 locus, in comparison to the centromeric region of the human chromosome X.

II. The REMBRANDT® LSI Xp22.31/CEP X- ISH detection assay is intended for the detection of the Xp22.31 locus, in comparison to the centromeric region of the human chromosome X in fixed cells. A clinical diagnosis should not be established based on the performance of this test.

III. The intended users are qualified laboratory employees in cytology and/or pathology. The product is intended for professional use.

Clinical relevance

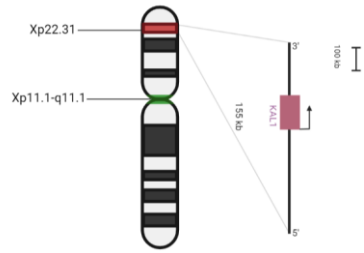
The Xp22.31 microdeletion syndrome, also known as the X-linked Kallmann syndrome KAL1. In female, the syndrome is caused by a heterozygous deletion of Xp22.31. In male, the syndrome is caused by a hemizygous deletion of Xp22.31, due to the lack of 2 X-chromosomes. The Kallmann syndrome has a prevalence in female of about 1 in 40,000 and in male of about 1 in 8,000 births worldwide (Dodé & Hardelin, 2009). KAL1 is characterized by the inability to start puberty, hypogonadism and bimanual synkinesis, in which the movements of one hand are mirrored by the other hand (Balasubramanian & Crowley, 1993). The KAL1 (Kallmann syndrome 1 sequence) gene, also known as the ANOS1 (anosmin 1) gene, is responsible for the X-linked form of KS (AbuJbara et al., 2004).

The ANOS1 gene has a pseudogene on the Y chromosome called ANOS2P, which is a nonfunctional gene that resembles the functional ANOS1 gene (de Castro et al., 2017; Dodé & Hardelin, 2009) .Rembrandt KAL1 FISH probes are designed to target the KAL1 critical region, mapping the ANOS1 gene of 155 kb long.

Probe specification

The REMBRANDT® LSI Xp22.31/CEP X probe set consists of a 155 kb probe detecting the Xp22.31 locus and a 0.68 kb centromeric probe detecting the centromeric region of chromosome X.

The centromeric region is conjugated to biotin and the locus is conjugated to digoxigenin. The LSI Xp22.31/CEP X-ISH assay is able to detect the Xp22.31 locus, in comparison to the centromeric region of the human chromosome X by means of direct *in situ* hybridization. The REMBRANDT® LSI Xp22.31/CEP X probes are pre-mixed in a hybridization mixture (formamide, dextran sulphate and SSC) and are ready to use solutions.



Test principle

In an *in situ* hybridization assay, a double stranded DNA probe labelled with a hapten is used. The labelled DNA probe is diluted in a hybridization mixture. The hybridization mixture containing the DNA probe is added to the specimen. A co-denaturation will ensure that the genomic DNA and the probe DNA become single stranded. After denaturation, the probe DNA is able to hybridize to its complementary target sequences in the cells. In the Rembrandt® LSI Xp22.31/CEP X-ISH detection assay, the haptens are attached to the probe and the signals can be visualized after detection by corresponding antibodies by fluorescent or brightfield microscopy.

Reagents provided

Product name	Product number	Amount
Labelled probes (depending on label and size choice)		
●LSI Xp22.31/CEP X-ISH probes biotin/digoxigenin detection	C803P.0199.05	Σ 5 T
●LSI Xp22.31/CEP X-ISH probes biotin/digoxigenin detection	C803P.0199.10	Σ 10 T
Pepsin powder	R011R.0000	1 g
Pepsin diluent	R018R.0000	15 ml
PanWash 4, 25X SSC	R025R.0000	15 ml

Assay procedure

REMBRANDT® LSI Xp22.31/CEP X-ISH detection assay procedure for cytological specimen.

I. Incubate slides in pre-heated proteolytic work solution (prepare according to section 1.9 of Manual-FISH) (R011R.000 + R018R.000) at 37 ˚C (100 µg/ml) for 15 minutes followed by a brief rinsing in 0.01M HCl (1x 2 minutes) and subsequent rinses in PBS (2x 1 minute)

II. Flush wash slides in deionised water, followed by dehydration in graded ethanol series (ethanol 70%, 96%, 96%, 100%, 100%) 1 minute each and air-dry slides for 15 minutes

Do not treat more than 5 slides at the same time, because the temperature of the pre-heated solutions may drop dramatically, thus causing incomplete pre-treatment. Additionally, allow the slides to air-dry as recommended; otherwise sections will be lost.

III. Homogenize probe solution (C803P.0199.YY) a spin briefly. Apply 15 µl of probe solution to each specimen. Cover all specimens with a cover slip (avoid air bubbles). Denature slides on a 80 ˚C hotplate or other heating device for 3 minutes

Work in a pre-set order to ensure that all slides have been incubated at 80 ˚C for the exact same time. Do not denature more than 5 slides at the same time, the temperature of the heating device may drop dramatically, thus causing incomplete denaturation.

IV. Transfer the slides into a moist environment and incubate for 16 hours at 37 ˚C.

V. Remove coverslips by soaking the slides in PBS at room temperature

VI. Incubate the slides in diluted, pre-heated PanWash 4 (R025R.000) (prepare according to section 1.9 of Manual-FISH)

Do not incubate more than 5 slides at the same time in PanWash 4, the temperature of the PanWash 4 may drop dramatically, causing wrong stringency conditions.

I. Appropriate detection system should be evaluated by the end-user. Recommended detection systems are listed below

Digoxigenin detection	Biotin detection
R003R.0000 Sheep aDig-AP conjugate	R041R.0000 Goat aBio-AP Fab conjugate
R004R.0000 Sheep aDig-HRP conjugate	R042R.0000 Goat aBio-HRP Fab conjugate

Interpretation of results

Hybridization of the LSI Xp22.31/CEP X probes is viewed using a fluorescence of brightfield microscope equipped with appropriate excitation and emission filters. Allowing visualization of a signal concentrated at the Xp22.31 locus of chromosome X in combination with signals representing the centromeric region of chromosome X. The enumeration Xp22.31 copy numbers conducted by microscopic examination of interphase nuclei, compared to the signals representing chromosome X.

The assay results are reported as the percentage of nuclei with 0, 1, 2, 3, 4, and >4 signals.

Enumerate the signals in the interphase nucleus using a 40X or 63X magnification. Objectives with higher magnification (eg, 63X or 100X) should be used to verify or resolve questions about split or diffused signals. Enumerate at least 100 nuclei per slide for accurate analysis.

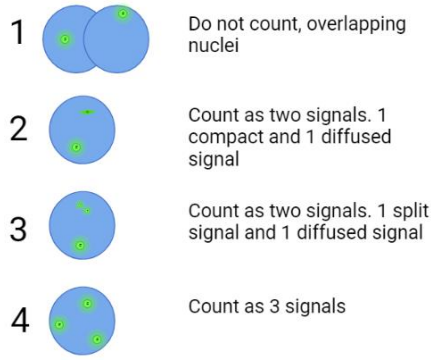
•Two signals in close proximity and approximately the same sizes but not connected by a visible link are counted as 2 signals.

•Count a diffuse signal as 1 signal if diffusion of the signal is contiguous and within an acceptable boundary.

•Two small signals connected by a visible link are counted as 1 signal.

•Enumerate the number of nuclei with 0, 1, 2, 3, 4, or >4 signals. Count nuclei with 0 signals only if there are other nuclei with at least 1 signal present in the field of view. If the accuracy of the enumeration is in doubt, repeat the enumeration in another area of the slide.

•Do not enumerate nuclei with uncertain signals



Performance characteristics

The REMBRANDT® LSI Xp22.31/CEP X-ISH detection assay was analytically validated for LSI Xp22.31/CEP X-FISH detection. The results of the direct fluorescent assay are shown. However, for the REMBRANDT® LSI Xp22.31/CEP X-ISH detection assay, the detection system may influence the performance characteristics and LSI Xp22.31/CEP X-ISH detection assay in combination with different detection systems should be evaluated carefully by the end-user.

Analytical sensitivity

The analytical sensitivity was determined in three levels. The normal cut-off percentage was determined based on assessment of 200 individual nuclei in two samples of healthy interphase lymphocytes from peripheral blood. The beta inversion was used to determine the percentage of normal-cut off. For the noise-to-signal percentage, the noise and signal values were determined for 100 signals in interphase lymphocytes from peripheral blood of two independent samples. The differences between noise and signal were evaluated using a 95% confidence interval. For the hybridization efficiency, 200 individual nuclei were assessed for the presence of ISH signals.

Performance characteristic	Outcome
Normal cut-off percentage	9.8%
Noise-to-signal cut-off percentage	25.4%
Hybridization efficiency	99%

Analytical specificity

The analytical specificity was determined in two levels. The theoretical specificity was determined by sequencing analysis of the probe DNA and mapping on Hg38. The practical specificity was determined by assessing the hybridization pattern in metaphase chromosomes of interphase lymphocytes from peripheral blood.

Performance characteristic	Outcome
Theoretical specificity	Mapped on chromosome X, p22.31 and the centromeric region Xp11.1-q11.1
Practical specificity	100%

Limitations of Procedure

i) The LSI Xp22.31/CEP X-ISH detection assay is solely applicable detection of the Xp22.31 locus, in comparison to the centromeric region of the human chromosome X, which may be present in cell preparations (cytological specimen i.e. interphase lymphocytes from peripheral blood samples).

ii) Either human tissue sections or human cytological preparations may be used. Samples must be fixed in buffered formalin or alcohol. In tissue sections are required, the sections should be prepared in a 4 µm thickness. Furthermore, the tissues should be glued to the glass slides with a bio-adhesive (e.g. organ silane), dried at room temperature, subsequently dried at 37 °C overnight and lastly completely deparaffinized in xylene and alcohol series and air dried.

iii) 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.

iv) 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.

v) The performance of the ISH procedure is also affected by the sensitivity of the method and the presence of the Xp22.31 locus and the centromeric region of chromosome X. In case the limit of the sensitivity is reached a false negative reaction may be the result.

vi) The LSI Xp22.31/CEP X-ISH detection assay results should not be relied on in case the sampling, sampling method, sample quality, sample preparation, reagents used, controls and procedure followed are not optimal or as described the working protocols.

ix) The medical profession should be aware of risks and factors influencing the fluorescent signal intensity while interpretating the test result. Microscopy settings might influence the signal intensity and/or interpretation.

x) Laboratory personnel performing the test should be trained and knowledgeable to be able to interpret the test results.

Storage and handling

Store kit and its contents at 2-8°C. Store the dissolved and aliquoted reagents at recommended temperatures. When used and stored as indicated, the kit is stable until the expiry date printed on the box.

Product	Product number	Storage conditions
LSI Xp22.31/CEP X-ISH probes biotin/digoxigenin detection	C803P.0199	2-8 ˚C
PanWash 4, 25X SSC	R025R.0000	Concentrated solution and diluted: 2-25˚C, ambient temperature
Pepsin powder	R011R.0000	Powder: 2-25˚C,

		ambient temperature
Pepsin diluent	R018R.0000	Dissolved: -20˚C Concentrated solution and diluted: 2-25˚C, ambient temperature

Hazard statements

H315 - Causes skin irritation
H319 - Causes serious eye irritation
H351 - Suspected of causing cancer
H360D - May damage the unborn child
H373 - May cause damage to organs through prolonged or repeated exposure

Precautionary Statements

P202 - Do not handle until all safety precautions have been read and understood
P280 - Wear protective gloves/protective clothing/eye protection/face protection
P302 + P352 - IF ON SKIN: Wash with plenty of water and soap
P305 + P351 + P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
P308 + P313 - IF exposed or concerned: Get medical advice/attention
P362 + P364 - Take off contaminated clothing and wash it before reuse
P405 - Store locked up

Additional information

Product in combination with other devices

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For additional information regarding the Rembrandt® assays, a manual is included which specifies the following subjects:

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