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I. Introduction

Best practice recommendations for high-throughput sequencing agree on the necessity of having an identity verification technique based on comparing the genotypes of several polymorphisms. These genotypes are obtained independently through next generation sequencing (NGS) and a second orthogonal technique. **SNPXplex** is a sample identity verification kit for simultaneous genotyping of 15 single nucleotide polymorphisms (SNPs) by allele-specific fluorescent multiplex PCR and sex determination.

II. Content

Reagent: **SNPXplex – Reference 99901.**

Volume: 1 200 µl, suitable for up to 130 reactions under optimal usage conditions.

III. Storage condition

Reagents should be stored at -20°C.

Reagents can be stored at room temperature for up to 8 days, protected from light.

IV. Instruments and reagents not supplied

The kit has been previously tested and validated on the following thermal cyclers:

- Eppendorf Vapo.Protect Mastercycler, MastercyclerX50a, Nexus
- Biometra T.Professional
- Life Technologies -Veriti
- Peqstar (VWR)
- Applied Biosystems SimpliAmp, 2700, 9700
- PeqLab, profex
- Biorad c1000, CFX96.

Capillary sequencer for fluorescent label detection [6Fam]

Fragment size analysis software or application

Formamide HiDi™

Size marker (recommended: GeneScan™ 400HD ROX™).

Alternative size marker such as GeneScan™ 600 LIZ™ may be used, ensuring that the sequencer is appropriately set to analyze the fluorochrome of the size marker.

V. Modification log

Version V7.3, 26 may 2025 : Minor text modifications.

Version V7.2, September 5, 2024: Added the list of interfering SNPs (genome version: GRCh37, in addition to GRCh38) – minor modification.

Version V6, April 25, 2024: Added an additional interferent in Table No. VIII.3 (major modification). Clarified starting matrices for SNPXplex analysis (major modification). Added additional validated capillary sequencers (minor modification).

VI. Protocol

Read thoroughly before use.

For any identity verification, it is **essential** that the orthogonal technique to NGS (in this case **SNPXplex**) is performed **independently** of the NGS method.

Please note that the sample rack used for one technique must not be used for the second. If this is the case, the manipulation becomes a method comparison and not an identity check.

1. Samples

List of extraction kits compatible and validated with the SNPXplex:

- NucleoSpin Blood kit (Macherey Nagel) (200 - 400 ng/μL DNA)
- Nucleon Bacc3 (Cytivia) (100 - 1000 ng/μL DNA)
- NucleoSpin 8 BLOOD (Macherey Nagel) (100 - 150 ng/μL DNA)
- Maxwell16 (50 - 200 ng/μL DNA)
- Chemagic extraction (PerkinElmer) (50 - 200 ng/μL DNA)
- QIAasymphony (80 - 200 ng/μL DNA)
- Wizard Promega (Precipitation) (22 - 500 ng/μL DNA)
- Manual extraction with Macherey Nagel Nucleopin Tissue kit (20 - 80 ng/μL DNA)
- QIAasymphony - Maxwell (20 - 400 ng/μL DNA)

☞ **Compatibility for starting samples: The SNPXplex kit has also been validated on DNA extracted from cell cultures, blood, plasma, FFPE tissue**

2. PCR

2.1. Volume to be dispensed per well

SNPXplex	9 μL*
DNA (stock solution)	1 μL**
<i>Final volume:</i>	<i>10 μL</i>

* In the event of failure with 9μL, the reagent volume can be increased to 24 μL without increasing the DNA volume.

** Recommended DNA quantity for PCR: For 9 μl of reagent: 0.5 to 700 ng/μL of DNA.

☞ **Be extremely cautious when sealing PCR tubes/plates.**

2.2. PCR cycles

Initial denaturation	95°C	2 min	
Denaturation	95°C	30 s	30 cycles
Hybridization	65°C	45 s	
Elongation	72°C	90 s	
Final extension	72°C	10 min	
Hold	10°C	∞	

3. Sequencer migration

3.1. PCR product dilution

Dilute the PCR products in sterile water (initial dilution recommendation: 1/50, to be adjusted locally).

3.2. Preparation of deposit mix

Add to 1 μl of the previous dilution 15 μl of a mixture composed of:

	Volume
Formamide HiDi™	15 μL
GeneScan™ 400HD ROX™	0.1 μL

3.3. Migration parameters

For Applied Biosystems® 3730/3730xl DNA Analyzer (Thermo Fisher), SeqStudio (Thermo Fisher), and Spectrum Compact CE System (Promega):

Oven temperature	66°C
Injection time	To be determined locally
Pre-run voltage	15kV
Injection voltage	2kV
Dye Set Fluorochromes	Any4Dye-HDR (or other Dye Set including 6-Fam and size marker fluorochrome)

Other sequencers: parameters must be determined by user.

4. Raw data analysis

To obtain analysis parameters for GeneMapper™ Software V5.1 or V6.0 (Applied Biosystems), contact support@bioxal.com.

For analysis using other software: follow the recommendations provided by the respective software publisher.

5. Comparison of SNPXplex vs NGS genotyping results and Genoidentity testing

SNPXplex genotyping data can be exported via the Export function of GeneMapper™.

Use local resources to:

- Compare patient by patient the **SNPXplex** genotyping results with those obtained by next generation sequencing (NGS).
- Search for possible genoidentity **between two patients in a series**.

VII. Informative value

The probability that two patients in a series of 96 share the same genotype for the 15 SNPs, based on the GnomAD frequencies of the analyzed SNPs and according to populations: African/African-American [0.004113]; East Asian [0.035343]; European (Finnish) [0.002426]; European (Non-Finnish) [0.002146]; Latino/Admixed American [0.004324]; South Asian [0.006360].

Determining gender reduces this risk by a factor n of at most 2, depending on the sex ratio in the series (n=2 if the sex ratio in the series is 1:1; n=1 if the sex ratio is totally unbalanced).

VIII. Support

For any issues or further information, please contact: support@bioxtal.com.

IX. Troubleshooting

1. Problems affecting peak intensity

Report	Possible cause(s)	Procedure
Intensity too low for all peaks for most of the samples	<ul style="list-style-type: none"> Excessive dilution of PCR product Injection time on sequencer too short PCR conditions not optimal 	<ul style="list-style-type: none"> Do not attempt to interpret Either place the same plate back on the capillary sequencer with a longer injection time (NB: proportionality between injection time and intensity / no proportionality between dilution factor and intensity). Alternatively, use a lower dilution of PCR products. Either repeat the SNPXplex
Low intensity for all peaks in one to several samples	<ul style="list-style-type: none"> Low quality DNA for these samples DNA too concentrated for these samples 	<ul style="list-style-type: none"> Do not attempt to interpret these samples Either perform identity vigilance based on a single variant of the NGS run for each sample concerned (threshold n to be determined locally) Or repeat the SNPXplex with 24µl of reagent + 1µl of DNA
High intensity for all peaks in most of the samples	<ul style="list-style-type: none"> PCR product too concentrated Sequencer injection time too long 	<ul style="list-style-type: none"> Do not attempt to interpret Re-do the same plate on the capillary sequencer with a shorter injection time (NB: proportionality between injection time and intensity / there is no proportionality between dilution factor and intensity). Alternatively, use a higher dilution of PCR products.
Heterogeneous intensity in the run	<ul style="list-style-type: none"> Tube capping/plate sealing not optimal Reaction volume <9µl 	<p>⇒ Ensure optimal sealing of PCR tubes.</p> <ul style="list-style-type: none"> Do not attempt to interpret samples with too low or too high intensity For strong samples: <ul style="list-style-type: none"> Place the same plate back on the sequencer with a shorter injection time Then repeat the comparison with the NGS results, using both SNPXplex runs For those too weak: <ul style="list-style-type: none"> Repeat the SNPXplex or perform identity vigilance using a single variant from the run
Absence of peaks for 1 or more SNPs for 1 or more samples	<ul style="list-style-type: none"> Non-optimal PCR conditions DNA quality too low DNA too concentrated 	<ul style="list-style-type: none"> Do not attempt to interpret the SNP(s) concerned Considering only the SNPs remaining interpretable (n<15) for all the samples in the series, is there an absence of genoidentity for all these samples? <ul style="list-style-type: none"> Yes ⇒ identity verification can be validated for the whole series No ⇒ <ul style="list-style-type: none"> Either perform identity verification using a unique variant of the NGS run for each sample concerned Or repeat the SNPXplex with 24µl of reagent + 1 µl of DNA for each concerned sample

2. Discordances*

Constat	Cause(s) possible(s)	Procedure
Discordance on several SNPs	⇒ DNAs tested under the same identifier in NGS and SNPXplex are not identical <ul style="list-style-type: none"> • Inversion of two samples? • Sample error? 	<ul style="list-style-type: none"> • Analyze the discordances to determine the source of the problem • Repeat <i>SNPXplex</i> or perform identity vigilance on a unique variant of the run
Discordance on a single SNP: <ul style="list-style-type: none"> • SNP heterozygous in NGS and homozygous in <i>SNPXplex</i> • SNP homozygous in NGS and heterozygous in <i>SNPXplex</i> • Peak(s) in an unexpected position 	SNPXplex is based on: <ul style="list-style-type: none"> • Allele-specific PCR • Genotype determination based on PCR product size It is therefore sensitive to anything that might interfere with PCR <ul style="list-style-type: none"> • with PCR: a variant other than that sought under one of the primers ⇒ prevents normal hybridization of the primer to its target sequence • with the size of PCR products: deletion or insertion variants between the two primers ⇒ modify the expected size of the PCR product. 	<ul style="list-style-type: none"> • Watch the bam files for a variant** that could interfere with the <i>SNPXplex</i> result. <ul style="list-style-type: none"> ○ If an interfering variant explaining the discrepancy is identified, the NGS results can be validated ○ If no cause is identified, perform identity vigilance using a single variant from the NGS run for each sample concerned **Refer to the table on the next page for a list of variants known to interfere with <i>SNPXplex</i> .

* For satisfactory NGS and SNPXplex quality

Batch # XP44.G.03

Production Date: 01/12/2025. Release Date: 01/12/2025. Expiration Date: 01/05/2026

Tableau VIII.3.1 – List of the studied SNPs and list of variants known to interfere with the SNPplex (genome version: GRCh37)**

SNPXplex SNPs					Observed Interference			
Rank (theoretical size ref-alt bp)	SNPXplex rs	Chr	GRCh37 coordinates	Alleles (ref/alt)	SNPXplex allele observed by NGS	GRCh37 genomic coordinates of the interfering variant	GnomAD (v2.1.1) Frequency of the interfering variant [min – max population]	Type of interference*** - Observed Effect on SNPplex
1 (103-106)	rs11702450	21	g.47703649	G/A	G	g.47703586C>T	-	H - Absence of amplification of the G allele
2 (111-114)	rs843345	3	g.183906515	T/C	T	g.183906517C>T	0.00002805 [0 - 0.00006212 NFE]	H - Absence of amplification of the T allele
3 (119-122)	rs1058018	17	g.47000251	C/T	C	g.47000252G>A	0.00003990 [0 - 0.00006173 AF]	H - Absence of amplification of the C allele
					T	g.47000164_47000185del	-	H - Absence of amplification of the T allele
						g.47000184_47000187del	0.0006751 [0 - 0.001350 SA]	T – T allele peak shift of -4 bp, in the allele C bin
4 (127-130)	rs8017	16	g.2821573	C/T	C	g.2821566_2821567del	0.0001944 [0 - 0.0006206 SA]	H - Absence of amplification of the C allele
						g.2821629_2821631del	0.00004382 [0 - 0.0002025 Latino]	T – C allele peak shift of -3 bp, outside any bin
					T	g.2821665G>A	-	H - Absence of amplification of the C allele
						g.2821566_2821567del	0.0001944 [0 - 0.0006206 SA]	H - Absence of amplification of the T allele
5 (135-138)	rs3738494	1	g.43124859	C/T	C	g.2821570del	-	H - Absence of amplification of the T allele
						g.2821658T>C	-	H - Absence of amplification of the T allele
					T	g.43124952C>T	0.00004949 [0.00 - 0.00008514 NFE]	H - Absence of amplification of the C allele
6 (143-146)	rs1065483	17	g.5284770	G/A	No known interfering variant	g.43124953C>T	-	H - Absence of amplification of the T allele
						g.43124859_43124862delinsT	-	T – T allele peak shift by -3 bp, in the C allele bin
7 (151-154)	rs2839181	21	g.47685939	A/G	A	g.47685932C>A	0.000007103 [0 - 0.00001550 NFE]	H - Absence of amplification of the A allele
						g.47685933A>G	0.000003997 [0 - 0.00005442 EA]	H - Absence of amplification of the A allele
						g.47685936G>A	-	H – Absence of amplification of the A allele
						G	g.47685925C>G	0.000004003 [0 - 0.000008809 NFE]
g.47685933_47685934del	0.00001998 [0 - 0.00005441 EA]	H - Absence of amplification of the G allele						
8 (159-162)	rs11059924	12	g.129293346	C/T	No known interfering variant			
9 (167-170)	rs2075144	19	g.46857286	G/A	No known interfering variant			

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SNPXplex SNPs					Observed Interference			
Rank (theoretical size ref-alt bp)	SNPXplex rs	Chr	GRCh37 coordinates	Alleles (ref/alt)	SNPXplex allele observed by NGS	GRCh37 genomic coordinates of the interfering variant	GnomAD (v2.1.1) Frequency of the interfering variant [min - max population]	Type of interference*** - Observed Effect on SNPXplex
10 (172-178)	rs6795772	3	g.49365269	C/T	T	g.49365145_49365148del	-	H - Absence of amplification of the T allele
						g.49365280G>A	0.00004838 [0 - 0.0001660 Other]	H - Absence of amplification of the T allele
11 (183-186)	rs456261	6	g.33258443	G/A	G	g.33258320_33258321del	0.000065 [0 - 0.0001334 NFE]	H - Absence of amplification of the G allele
					A	g.33258411_33258414del	-	T - A allele peak shift of -4 bp, in the G allele bin
12 (191-194)	rs1131620	19	g.41117869	A/G	A	g.41117710C>T	0.004342 [0 - 0.08419 EA]	H - Absence of amplification of the A allele
						g.41117716G>A	0.0002268 [0 - 0.001847 SA]	H - Absence of amplification of the A allele
						g.41117870C>G	0.00003185 [0 - 0.00006481 NFE]	H - Absence of amplification of the A allele
					G	g.41117886C>T	0.002784 [0 - 0.03111 AA]	H - Strong peak intensity decrease of the G allele
14 (207-210)	rs2231926	3	g.73111809	A/G	G	g.73111825G>C	-	H - Absence of amplification of the G allele
						g.73111828A>G	0.0008997 [0 - 0.004163 AshJ]	H - Peak intensity decrease of the G allele
15 (215-218)	rs352169	3	g.52236762	G/A	G	g.52236739_52236740delinsAA	-	H - Absence of amplification of the G allele
X (224)	Amplicon of the UBL4A gene (ChrX) used for sex determination				-	g.153713745_153713758delinsTGACACA	-	T - X peak shift of -6 bp in the rs352169 bin
					-	g.153713811_153713813del	0.00001107 [0 - 0.00002490]	T - X peak shift of -3 bp outside any bin
Y (227)	Amplicon of the SRY gene (ChrY) used for sex determination				No known interfering variant			
16 (240-243)	rs3739160	2	g.105654716	C/T	C	g.105654872_105654874del	-	T - C allele peak shift of -3 bp, outside any bin
						g.105654850_105654860del	0.0061496 [0 - 0.01051 NFE]	T - T allele peak shift of -11 bp. NB: a bin for each allele (C or T) is provided for this purpose (cf profile example on page 10)
					T	g.105654700G>C	0.002605 [0 - 0.02738 AF]	H - Absence of amplification of the T allele
						g.105654710G>T	0.0008594 [0 - 0.009135 AF]	H - Absence of amplification of the T allele
						g.105654831del	-	T - T allele peak shift of -1 bp

**List established based on user experience. Variants interfering with the SNPXplex other than those listed may be highlighted. Please report them in order to enrich the list.

*** H: Interference on hybridization of one of the primers; T: interference on the size of the PCR product

In yellow : Latest modifications of the table (<6 months).

Batch # XP44.G.03

Production Date: 01/12/2025. Release Date: 01/12/2025. Expiration Date: 01/05/2026

Tableau VIII.3.2 – List of the studied SNPs and list of variants known to interfere with the SNPXplex (genome version: GRCH38)**

SNPXplex SNPs					Observed Interference			
Rank (theoretical size ref-alt bp)	SNPXplex rs	Chr	GRCh38 coordinates	Alleles (ref/alt)	SNPXplex allele observed by NGS	GRCh38 genomic coordinates of the interfering variant	GnomAD (v4.1.0) Frequency of the interfering variant [min – max population]	Type of interference*** - Observed Effect on SNPXplex
1 (103- 106)	rs11702450	21	g.46283735	G/A	G	g.46283672C>T	0.000001859 [0 – 0.000002542 NFE]	H - Absence of amplification of the G allele
2 (111- 114)	rs843345	3	g.184188727	T/C	T	g.184188729C>T	0.00001924 [0 - 0.00003266 Rem]	H - Absence of amplification of the T allele
3 (119- 122)	rs1058018	17	g.48922889	C/T	C	g.48922890G>A	0.000003990 [0 - 0.00006173 AF]	H - Absence of amplification of the C allele
					T	g.48922802_48922823del	-	H - Absence of amplification of the T allele
						g.48922822_48922825del	0.0006751 [0 - 0.001350 SA]	T – T allele peak shift of -4 bp, in the allele C bin.
4 (127- 130)	rs8017	16	g.2771572	C/T	C	g.2771565_2771566del	0.0001944 [0 - 0.0006206 SA]	H - Absence of amplification of the C allele
						g.2771628_2771630del	0.00004382 [0 - 0.0002025 Latino]	T – C allele peak shift of -3 bp, outside any bins
					T	g.2771664G>A	-	H - Absence of amplification of the C allele
						g.2771565_2771566del	0.0001944 [0 - 0.0006206 SA]	H - Absence of amplification of the T allele
5 (135- 138)	rs3738494	1	g.42659188	C/T	C	g.2771569del	-	H - Absence of amplification of the T allele
						g.2771657T>C	-	H - Absence of amplification of the T allele
					T	g.42659281C>T	0.00004949 [0.00 - 0.00008514 NFE]	H - Absence of amplification of the C allele
6 (143- 146)	rs1065483	17	g.5381475	G/A	No known interfering variant	g.42659282C>T	-	H - Absence of amplification of the T allele
						g.42659188_42659191delinsT	-	T – T allele peak shift of -3 bp, in the C allele bin
7 (151- 154)	rs2839181	21	g.46266025	A/G	A	g.46266018C>A	0.000007103 [0 - 0.00001550 NFE]	H - Absence of amplification of the A allele
						g.46266019A>G	0.000003997 [0 - 0.00005442 EA]	H - Absence of amplification of the A allele
						g.46266022G>A	-	H – Absence of amplification of the A allele
					G	g.46266011C>G	0.000004003 [0 - 0.000008809 NFE]	H - Absence of amplification of the G allele
g.46266019_46266020del	0.00001998 [0 - 0.00005441 EA]	H - Absence of amplification of the G allele						
8 (159- 162)	rs11059924	12	g.128808801	C/T	No known interfering variant			
9 (167- 170)	rs2075144	19	g.46354029	G/A	No known interfering variant			

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SNPXplex SNPs					Observed Interference			
Rank (theoretical size ref/alt bp)	SNPXplex rs	Chr	GRCh38 coordinates	Alleles (ref/alt)	SNPXplex allele observed by NGS	GRCh38 genomic coordinates of the interfering variant	GnomAD (v4.1.0) Frequency of the interfering variant [min – max population]	Type of interference*** - Observed Effect on SNPXplex
10 (172- 178)	rs6795772	3	g.49327836	C/T	T	g.49327712_49327715del	-	H - Absence of amplification of the T allele
						g.49327847G>A	0.00004838 [0 - 0.0001660 Other]	H - Absence of amplification of the T allele
11 (183- 186)	rs456261	6	g.33290666	G/A	G	g.33290543_33290544del	0.000065 [0 - 0.0001334 NFE]	H - Absence of amplification of the G allele
					A	g.33290634_33290637del	-	T – A allele peak shift of -4 bp, in the G allele bin
12 (191- 194)	rs1131620	19	g.40611963	A/G	A	g.40611804C>T	0.004342 [0 - 0.08419 EA]	H - Absence of amplification of the A allele
						g.40611810G>A	0.0002268 [0 - 0.001847 SA]	H - Absence of amplification of the A allele
						g.40611964C>G	0.00003185 [0 - 0.00006481 NFE]	H - Absence of amplification of the A allele
						g.40611980C>T	0.002784 [0 - 0.03111 AA]	H – Strong peak intensity decrease of the G allele
14 (207- 210)	rs2231926	3	g.73062658	A/G	G	g.73062674G>C	-	H - Absence of amplification of the G allele
						g.73062677A>G	0.0008997 [0 - 0.004163 AshJ]	H – Peak intensity decrease of the G allele
15 (215- 218)	rs352169	3	g.52202746	G/A	G	g.52202723_52202724delinsAA	-	H - Absence of amplification of the G allele
X (224)	Amplicon of the UBL4A gene (ChrX) used for sex determination				-	g.154485406_154485419delinsTGTACACA	-	T – X peak shift of -6 bp in the rs352169 bin
					-	g.154485472_154485474del	0.00001107 [0 - 0.00002490]	T – X peak shift of -3 bp outside any bin
Y (227)	Amplicon of the SRY gene (ChrY) used for sex determination				No known interfering variant			
16 (240- 243)	rs3739160	2	g.105038258	C/T	C	g.105038414_105038416del	;	T – C allele peak shift of -3 bp, outside any bin
						g.105038392_105038402del	0.0061496 [0 – 0.01051 NFE]	T – T allele peak shift of -11 bp. NB: a bin for each allele (C or T) is provided for this purpose (cf profile example on page 10)
						g.105038242G>C	0.002605 [0 - 0.02738 AF]	H – Absence of amplification of the T allele
						g.105038252G>T	0.0008594 [0 - 0.009135 AF]	H - Absence of amplification of the T allele
						g.105038373del	;	T – T allele peak shift of -1 bp

**List established based on user experience. Variants interfering with the SNPXplex other than those listed may be highlighted. Please report them in order to enrich the list.

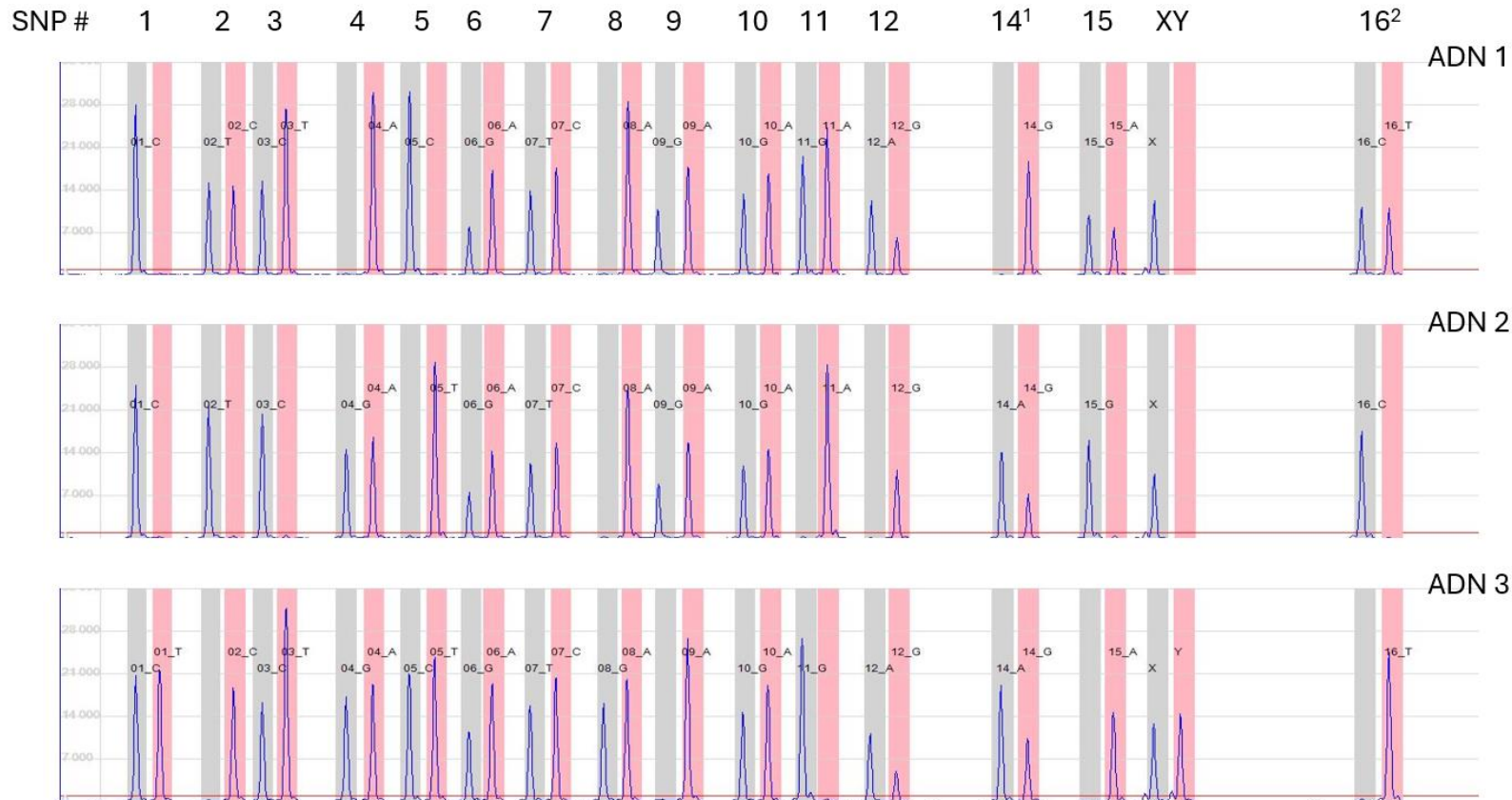
*** H: Interference on hybridization of one of the primers; T: interference on the size of the PCR product

In yellow : Latest modifications of the table (<6 months).

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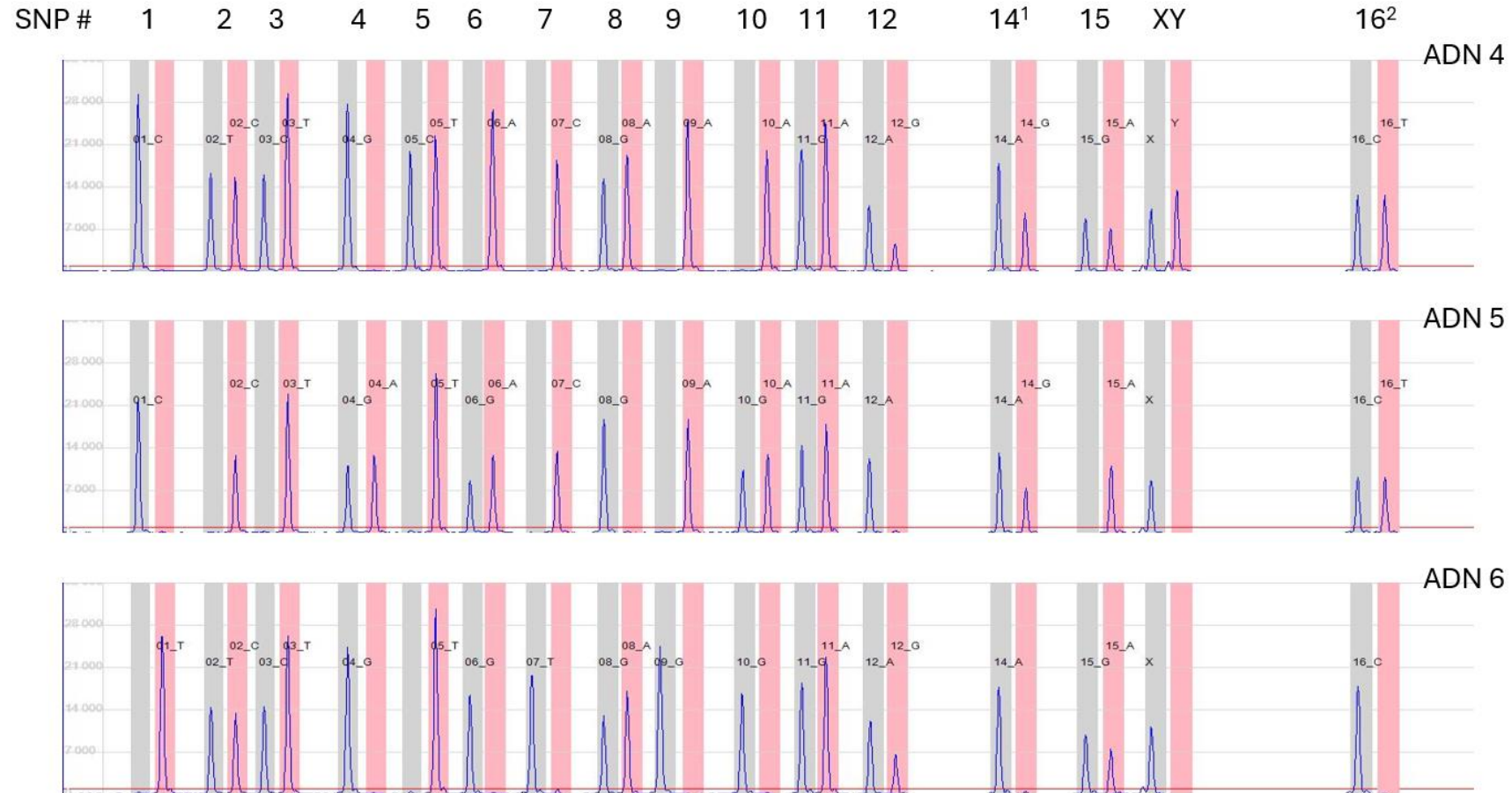
X. Example of profiles



In grey, the reference SNP, in pink, the alternative SNP.

¹There is no SNP#13

²For this SNP, rs368436190 (maximum frequency = 0.01067 in European [Non-Finnish]) leads to a deletion of 11 bp in the PCR product, hence the presence of 2 bins per allele, one for alleles not carrying rs368436190, the second for alleles carrying rs368436190



In grey, the reference SNP, in pink, the alternative SNP.









¹There is no SNP#13

²For this SNP, rs368436190 (maximum frequency = 0.01067 in European [Non-Finnish]) leads to a deletion of 11 bp in the PCR product, hence the presence of 2 bins per allele, one for alleles not carrying rs368436190, the second for alleles carrying rs368436190

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XI. Symbol identification

Symbol	Description
	This symbol indicates the address of the manufacturer.
	This symbol indicates the manufacturing date.
	Consult the user manual before use. A QR Code provides access to your notice.
	Expiration date. This symbol indicates the date after which the medical device must no longer be used.
	This symbol indicates the optimal storage temperature.
	This symbol indicates the manufacturer's catalog reference.
	This symbol identifies the lot number.
	This symbol indicates that the content is sufficient for “n” tests, in normal condition of use.