



BIOXTAL 227 Route de la Chapelle 74540 Saint Félix, France Standard: +33 (0)4 50 10 18 44 www.bioxtal.com

SARL with capital of €286.000

n° lot: XP30.D.03

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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 technique sequencing (NGS) and a second technique. **SNPXplex** is an 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

Volume: 1 200 µl, equivalent of 130 reactions under optimal usage condition.

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, Mastercycler X50a, 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

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

<u>Version V6, April 25, 2024</u>: 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).





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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.

<u>Please note that the sample rack used for one technique must not be used</u> <u>for the second.</u> 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)
- QIAsymphony (80 200 ng/µL DNA)
- Wizard Promega (Precipitation) (22 500 ng/µL DNA)
- Manual extraction with Macherev Nagel Nucleopin Tissue kit (20 80 ng/uL DNA)
- QIAsymphony 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**
Final volume	10 μL

 $^{^{\}star}$ In the event of failure with 9µL, the reagent volume can be increased to 24 µL without increasing the DNA volume.

Be extremely cautious when sealing PCR tubes/plates.

2.2. PCR cycles

Initial denaturation	95°C	2 min	
Denaturation	95°C	30 s	
Hybridization	65°C	3 min	30 cycles
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@bioxtal.com.

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

^{**} Recommended DNA quantity for PCR: For 9 μ l of reagent: 0.5 to 700 ng/ μ L of DNA.





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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.





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IX. Troubleshooting

1. Problems affecting peak intensity

Report	Possible cause(s)	Procedure
Intensity too low for all peaks for most of the samples	Excessive dilution of PCR product Injection time on sequencer too short PCR conditions not optimal	 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	Low quality DNA for these samples DNA too concentrated for these samples	 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	PCR product too concentrated Sequencer injection time too long	 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	Tube capping/plate sealing not optimal Reaction volume <9µl	 ⇒ Ensure optimal sealing of PCR tubes. Do not attempt to interpret samples with too low or too high intensity For strong samples: 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: 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	Non-optimal PCR conditions DNA quality too low DNA too concentrated	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?





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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 Inversion of two samples? Sample error? 	 Analyze the discordances to determine the source of the problem Repeat SNPXplex or perform identity vigilance on a unique variant of the run
Discordance on a single SNP:	SNPXplex is based on: Allele-specific PCR	Watch <i>the bam files</i> for a variant** that could interfere with the <i>SNPXplex</i> result.
SNP heterozygous in NGS and homozygous in SNPXplex	Genotype determination based on PCR product size	 If an interfering variant explaining the discrepancy is identified, the NGS results can be validated
SNP homozygous in NGS and heterozygous in SNPXplex	 It is therefore sensitive to anything that might interfere with PCR 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 	o 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
Peak(s) in an unexpected position	two primers ⇒ modify the expected size of the PCR product.	SNPXplex.

^{*} For satisfactory NGS and SNPXplex quality





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Tableau VIII.3.1 – List of the studied SNPs and list of variants known to interfere with the SNPXplex (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 SNPXplex											
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	Т	g.183906517C>T	0.00002805 [0 - 0.00006212 NFE]	H - Absence of amplification of the T allele											
					С	g.47000252G>A	0.000003990 [0 - 0.00006173 AF]	H - Absence of amplification of the C allele											
3	4050040	4-	47000054	0.77		g.47000164_47000185del	-	H - Absence of amplification of the T allele											
(119- 122)	rs1058018	17	g.47000251	C/T	Т	g.47000184_47000187del	0.0006751 [0 - 0.001350 SA]	T – T allele peak shift of -4 bp, in the allele C bin											
,						g.47000260C>T	-	H - Absence of amplification of the T allele											
						g.2821566_2821567del	0.0001944 [0 - 0.0006206 SA]	H - Absence of amplification of the C allele											
			g.2821573			С	g.2821629_2821631del	0.00004382 [0 - 0.0002025 Latino]	T – C allele peak shift of -3 bp, outside any bin										
4	0047	40		0.77		g.2821665G>A		H - Absence of amplification of the C allele											
(127- 130)	rs8017	16		g.2821573 C/T		g.2821566_2821567del	0.0001944 [0 - 0.0006206 SA]	H - Absence of amplification of the T allele											
100,					T	g.2821570del	-	H - Absence of amplification of the T allele											
																		g.2821658T>C	-
5			g.43124859 C/T		С	g.43124952C>T	0.00004949 [0.00 - 0.00008514 NFE]	H - Absence of amplification of the C allele											
(135-	rs3738494	1		g.43124859 C/T	g.43124859 C/T	C/T	O/T	C/T	C/T	C/T	T	g.43124953C>T	-	H - Absence of amplification of the T allele					
138)											!	g.43124859_43124862delinsT	-	T – T allele peak shift by -3 bp, in the C allele bin					
6 (143- 146)	rs1065483	17	g.5284770	G/A	No known interfering	g variant													
						g.47685932C>A	0.000007103 [0 - 0.00001550 NFE]	H - Absence of amplification of the A allele											
7					Α	g.47685933A>G	0.000003997 [0 - 0.00005442 EA]	H - Absence of amplification of the A allele											
(151-	rs2839181	21	g.47685939	A/G		g.47685936G>A	-	H – Absence of amplification of the A allele											
154)					G	g.47685925C>G	0.000004003 [0 - 0.000008809 NFE]	H - Absence of amplification of the G allele											
					G	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	g variant													
9 (167- 170)	rs2075144	19	g.46857286	G/A	No known interfering	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	0705770	•	40005000	O.T.	-	g.49365145_49365148del	-	H - Absence of amplification of the T allele														
(172- 178)	rs6795772	3	g.49365269	C/T	l	g.49365280G>A	0.00004838 [0 - 0.0001660 Other]	H - Absence of amplification of the T allele														
11	450004		~ 22050442	0/4	G	g.33258320_33258321del	0.000065 [0 - 0.0001334 NFE]	H - Absence of amplification of the G allele														
(183- 186)	rs456261	6	g.33258443	G/A	Α	g.33258411_33258414del	-	T – A allele peak shift of -4 bp, in the G allele bin														
						g.41117710C>T	0.004342 [0 - 0.08419 EA]	H - Absence of amplification of the A allele														
12	4404000	40	- 44447000	A/O	Α	g.41117716G>A	0.0002268 [0 - 0.001847 SA]	H - Absence of amplification of the A allele														
(191- 194)	rs1131620	19	g.41117869	A/G		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				3111809 A/G		g.73111825G>C	-	H - Absence of amplification of the G allele														
(207- 210)	rs2231926	3	g./3111809		./3111809 A/G	g./3111809 A/G	g.73111809 A/G	g./3111809 A/G	1809 A/G	G	g.73111828A>G	0.0008997 [0 - 0.004163 AshJ]										
15 (215- 218)	rs352169	3	g.52236762	G/A	G	g.52236739_52236740delinsAA	-	H - Absence of amplification of the G allele														
Х	Amplicon of t	he UBL	4A gene (ChrX) us	ed for sex	-	g.153713745_153713758delinsTGTACACA	-	T – X peak shift of -6 bp in the rs352169 bin														
(224)	'	det	ermination		-	g.153713811_153713813del	0.00001107 [0 - 0.00002490]	T – X peak shift of -3 bp outside any bin														
Y (227)	, p				No known interferin	g variant																
					0	g.105654872_105654874del	-	T – C allele peak shit of -3 bp, outside any bin														
16 (240-	rs3739160	2	g.105654716	C/T	С	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)														
243)	.55.56166	_	g555011 10	3/1	' _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 shit 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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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													
					С	g.48922890G>A	0.000003990 [0 - 0.00006173 AF]	H - Absence of amplification of the C allele													
3	rs1058018	17	~ 4000000	C/T		g.48922802_48922823del	-	H - Absence of amplification of the T allele													
(119- 122)	181000010	17	g.48922889	C/T	T	g.48922822_48922825del	0.0006751 [0 - 0.001350 SA]	T – T allele peak shift of -4 bp, in the allele C bin.													
ĺ						g.48922898C>T	-	H - Absence of amplification of the T allele													
						g.2771565_2771566del	0.0001944 [0 - 0.0006206 SA]	H - Absence of amplification of the C allele													
			g.2771572 C/T							j	С	g.2771628_2771630del	0.00004382 [0 - 0.0002025 Latino]	T – C allele peak shift of -3 bp, outside any bins							
4 (127-	rs8017	16		С/Т		g.2771664G>A	-	H - Absence of amplification of the C allele													
130)	150017	10			T	g.2771565_2771566del	0.0001944 [0 - 0.0006206 SA]	H - Absence of amplification of the T allele													
						g.2771569del	-	H - Absence of amplification of the T allele													
						g.2771657T>C	-	H - Absence of amplification of the T allele													
5	0700404		g.42659188 C/T	- 40050400 OT	- 40050400	- 40050400	- 40050400	- 400F0400 O/T	~ 40050400	40050400	40050400	40050400	40050400	- 40050400	~ 400F0400 C/T	~ 40650199 C/T	/2650188 C/T	С	g.42659281C>T	0.00004949 [0.00 - 0.00008514 NFE]	H - Absence of amplification of the C allele
(135- 138)	rs3738494	1		g.42059188 C/I	009100 C/I	.42009188 C/I	00 C/I	C/1	L/1	0/1	6/1	C/1	Т	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									
6 (143- 146)	rs1065483	17	g.5381475	G/A	No known interferin	g variant															
						g.46266018C>A	0.000007103 [0 - 0.00001550 NFE]	H - Absence of amplification of the A allele													
7					А	g.46266019A>G	0.000003997 [0 - 0.00005442 EA]	H - Absence of amplification of the A allele													
(151-	rs2839181	21	g.46266025	A/G		g.46266022G>A	-	H – Absence of amplification of the A allele													
154)					G	g.46266011C>G	0.000004003 [0 - 0.000008809 NFE]	H - Absence of amplification of the G allele													
					G	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 interferin	g variant															
9 (167- 170)	rs2075144	19	g.46354029	G/A	No known interferin	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	0705770		40007000	0.77	-	g.49327712_49327715del	-	H - Absence of amplification of the T allele												
(172- 178)	rs6795772	3	g.49327836	C/T	T	g.49327847G>A	0.00004838 [0 - 0.0001660 Other]	H - Absence of amplification of the T allele												
11					G	g.33290543_33290544del	0.000065 [0 - 0.0001334 NFE]	H - Absence of amplification of the G allele												
(183- 186)	rs456261	6	g.33290666	G/A	Α	g.33290634_33290637del	-	T – A allele peak shift of -4 bp, in the G allele bin												
·						g.40611804C>T	0.004342 [0 - 0.08419 EA]	H - Absence of amplification of the A allele												
12	rs1131620	19	g.40611963	A/G	Α	g.40611810G>A	0.0002268 [0 - 0.001847 SA]	H - Absence of amplification of the A allele												
(191- 194)	181131020	19		A/G		g.40611964C>G	0.00003185 [0 - 0.00006481 NFE]	H - Absence of amplification of the A allele												
,					G	g.40611980C>T	0.002784 [0 - 0.03111 AA]	H – Strong peak intensity decrease of the G allele												
14			70000050	g.73062658 A/G	g.73062658 A/G	g.73062658 A/G	g.73062658 A/G				g.73062658 A/G							g.73062674G>C	-	H - Absence of amplification of the G allele
(207- 210)		g./3062658 A/G	g./3062658 A/G					2658 A/G	A/G	G		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 Amplicon of the UBL4A gene (ChrX) used for sex			ed for sex	-	g.154485406_154485419delinsTGTACACA	-	T – X peak shift of -6 bp in the rs352169 bin													
(224)		det	ermination		-	g.154485472_154485474del	0.00001107 [0 - 0.00002490]	T – X peak shift of -3 bp outside any bin												
Y Amplicon of the SRY gene (ChrY) used for sex (227) determination				d for sex	No known interferin	g variant														
					С	g.105038414_105038416del	-	T - C allele peak shit of -3 bp, outside any bin												
16 (240-	rs3739160	2	q.105038258	С/Т	0	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 profile example on page 10)												
243)	.55. 56 166	_	g. 10000200	3/1	Т	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												
i						g.105038373del	-	T – T allele peak shit 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).

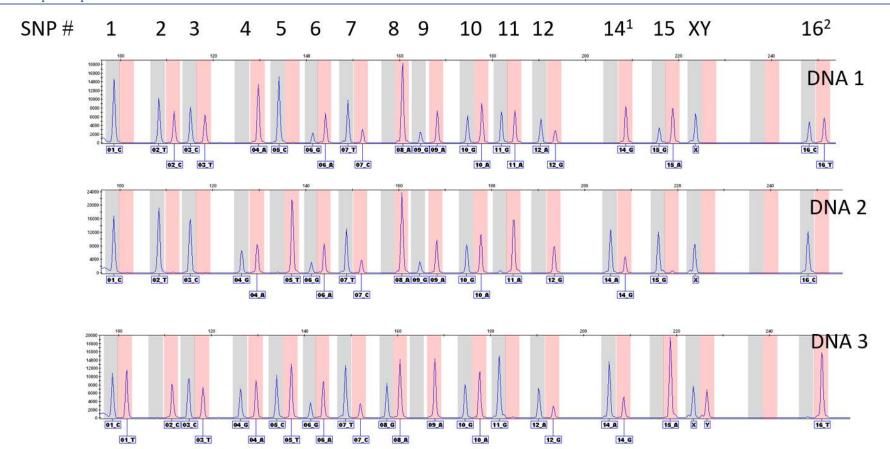
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X. Example of profiles



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

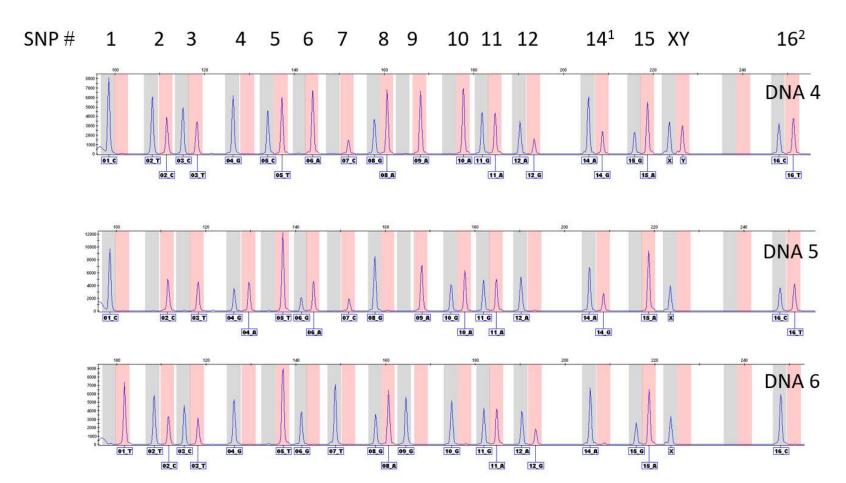
²For this SNP, rs368436190 (maximum frequency = 0.01067 in European [Non-Finnish]) leads to a deletion of 11 bp in the PCR product, hence the presence of 2 bins per allele, one for alleles not carrying rs368436190, the second for alleles carrying rs368436190

¹There is no SNP#13

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XI. Symbol identification

Symbol	Description
	This symbol indicates the address of the manufacturer.
	This symbol indicates the manufacturing date.
[]i	Consult the user manual before use. A QR Code provide access to your notice.
\square	Expiration date. This symbol indicates the date after which the medical device must no longer be used.
	This symbol indicates the optimal storage temperature.
REF	This symbol indicates the manufacturer's catalog reference.
LOT	This symbol identifies the lot number
Σ	This symbol indicates that the content is sufficient for "n" tests.