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I. Introduction

Good practice guidelines for high-throughput sequencing agree on the need for an identity verification technique, based on comparison of the genotypes of several polymorphisms, with the genotypes obtained independently by next-generation 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: 110 µl, the equivalent of 10 reactions under optimal conditions of use.

III. Preservation

Reagents can 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

HiDi Formamide

Size marker (recommended: GeneScan™ 400HD ROX™), it is possible to use another size marker such as GeneScan™ 600 LIZ™ taking care that the sequencer is configured to analyze the fluorochrome of the size marker.

V. Protocol

Please read carefully 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 technique.

Under no circumstances should the DNA array used in the NGS technique be used as is to run the SNPXplex technique (or vice versa): if this were done, it would be a method comparison, not an identity verification.

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)
- QIA Symphony (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)
- QIA Symphony - Maxwell (20 - 400 ng/μL DNA)

☞ **Compatibility for starting samples: The SNPXplex kit has also been validated directly on cell culture, blood, plasma, FFPE tissue**

2. PCR

2.1. Volume to be distributed per well

SNPXplex	9 μL*
DNA (stock solution)	1 μL**
Final volume	10 μL

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

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

☞ **Take extreme care when capping tubes/sealing PCR plates**

2.2. PCR cycles

Dénaturation initiale	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 mn	
Preservation	10°C	∞	

2. Sequencer migration

2.1. Dilution of PCR products

Dilute PCR products in sterile water (initially 1/50, to be adjusted locally).

2.2. Preparing the deposit mix

To 1 μl of the previous dilution, add 15 μl of a mixture composed of:

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

2.3. Migration parameters

On Applied Biosystems® 3730/3730xl DNA Analyzer (Life Technologies):

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 to be determined by user

3. Raw data analysis

To obtain analysis parameters on GeneMapper™ Software 5 software (Applied Biosystems), contact support@primadiag.com.

For analysis using other software: follow the publisher's recommendations.

4. Comparison of SNPXplex vs NGS genotyping results and genoidentity testing

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

Use local resources to:

- Compare **SNPXplex** genotyping results on a **patient-by-patient** basis with those obtained by high-throughput sequencing.
- Search for possible genoidentity **between two patients in a series**.



Production date :
19/10/23

SNPXplex[®]

Lot number: XP-25-C-02

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ZAC de l'Horloge – Building C1,
93230 ROMAINVILLE, France
Standard: +33 (0)1 48 44 04 16
www.primadiag.com
SAS with 609 024 euros of capital

VI. Informativity

Risk of two patients in a series of 96 carrying the same genotype for the 15 SNPs, based on the GnomAD frequencies of the SNPs analyzed 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).

VII. Support

In the event of a problem or for any additional information, please contact support@primadiag.com as a priority.

VIII. Troubleshooting

1. Problems affecting peak intensity

Report	Possible cause(s)	Procedure
Intensity too low for all peaks for a majority of samples	<ul style="list-style-type: none"> Excessive dilution of PCR product Injection time on sequencer too short PCR conditions not compliant 	<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
Intensity too low for all peaks for 1 to n samples	<ul style="list-style-type: none"> Lower 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
Intensity too high for all peaks for a majority of samples	<ul style="list-style-type: none"> Dilution of PCR products too low Injection time on sequencer too long 	<ul style="list-style-type: none"> Do not attempt to interpret Redose the same plate on the capillary sequencer with a shorter injection time (NB: proportionality between injection time and intensity / no proportionality between dilution factor and intensity). Alternatively, use a higher dilution of PCR products.
Heterogeneity of intensity in the run	<ul style="list-style-type: none"> Tube capping/plate sealing not optimal Reaction volume <9µl 	<p>⇒ Take extreme care when capping PCR tubes.</p> <ul style="list-style-type: none"> Do not attempt to interpret samples that are too weak or too strong 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 Lower quality DNA for these samples DNA too concentrated for these samples 	<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 ⇒ identityvigilance 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 sample concerned

2. Discordances*

Constat	Cause(s) possible(s)	Conduite à tenir
Discordance on several SNPs	<p>⇒ DNAs tested under the same identifier in NGS and SNPXplex are not identical</p> <ul style="list-style-type: none"> • Inversion of two samples? • Sample error? 	<ul style="list-style-type: none"> • Analyzing discrepancies can help us understand where the problem lies • Redo the <i>SNPXplex</i> or perform identity vigilance on a unique variant of the run
<p>Discordance on a single SNP:</p> <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> • Pic(s) in an unexpected position 	<p><i>SNPXplex</i> is based on:</p> <ul style="list-style-type: none"> • Allele-specific PCR • Genotype determination based on PCR product size <p>It is therefore sensitive to anything that might interfere with PCR</p> <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 bams 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 <p>**Refer to the table on the next page for a list of variants known to interfere with <i>SNPXplex</i>.</p>

* For satisfactory NGS and SNPXplex quality

List of SNPs studied and list of variants known to interfere with the *SNPXplex***

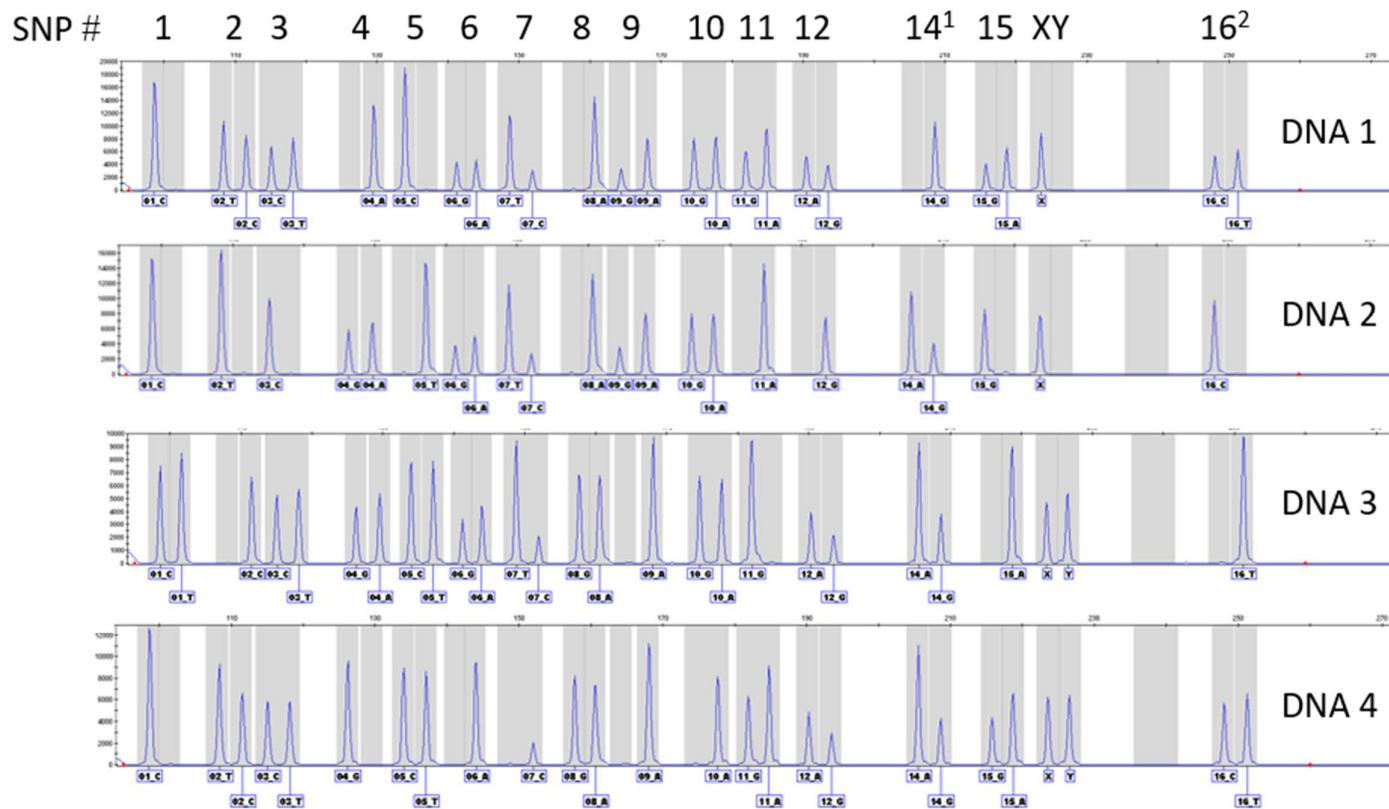
SNPXplex SNPs					Interference observed			
Rank (theoretical size ref-alt pb)	SNPXplex rs	Chr	Coordinates GRCh37	Alleles (ref/alt)	SNPXplex allele observed by NGS	GRCh37 genomic coordinates of the interfering variant	GnomAD frequency (v2.1.1) of the interfering variant [min – max population]	Type of interference*** - Effect observed on SNPXplex
1 (103-106)	rs11702450	21	g.47703649	G/A	No known interfering variant			
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 C allele 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.2821658T>C	-	H - Absence of amplification of the T allele
5 (135-138)	rs3738494	1	g.43124859	C/T	C	g.2821566_2821567del	0.0001944 [0 - 0.0006206 SA]	H - Absence of amplification of the T allele
						g.43124952C>T	0.00004949 [0.00 - 0.00008514 NFE]	H - Absence of amplification of the C allele
					T	g.43124859_43124862delinsT	-	T - T allele peak shift of -3 bp, in the C allele bin
						g.43124953C>T	-	H - Absence of amplification of the T allele
6 (143-146)	rs1065483	17	g.5284770	G/A	No known interfering variant			
7 (151-154)	rs2839181	21	g.47685939	A/G	A	g.47685936G>A	-	H - Absence of amplification of the A allele
						g.47685932C>A	0.00007103 [0 - 0.00001550 NFE]	H - Absence of amplification of the A 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 variant			
9 (167-170)	rs2075144	19	g.46857286	G/A	No known interfering variant			
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 d'amplification de l'allèle G
					A	g.33258411_33258414del	-	T - Peak shift of the A allele by -4 bp, in the G allele bin
12 (191-194)	rs1131620	19	g.41117869	A/G	A	g.41117710C>T	0.004568 [0 - 0.08467 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

SNPXplex SNPs					Interference observed			
Rank (theoretical size ref-alt pb)	SNPXplex rs	Chr	Coordinates GRCh37	Alleles (ref/alt)	SNPXplex allele observed by NGS	GRCh37 genomic coordinates of the interfering variant	GnomAD frequency (v2.1.1) of the interfering variant [min – max population]	Type of interference*** - Effect observed on SNPXplex
					G	g.41117886C>T	0.002784 [0 - 0.03111 AA]	H - Strong decrease in peak intensity of the G allele
14 (207-210)	rs2231926	3	g.73111809	A/G	G	g.73111828A>G	0.0008894 [0.00004 - 0.0042 AshJ]	H - Decreased peak intensity of the G allele
						g.73111825G>C	-	H - Absence of amplification 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 (ChrX) gene used for sex determination				-	g.153713745_153713758delinsTGTACACA	-	T - Shift of a peak X of -6 bp, in the bin of the A allele of rs352169
					-	g.153713811_153713813del	0.00001107 [0 - 0.00002490]	T - Offset of a peak X 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.105654850_105654860del	0.0061496 [0 - 0.01051 NFE]	T - Peak shift of the T allele by -11 bp. NB: a bin for each allele (C or T) is provided for this purpose.
					T	g.105654700G>C	0.002638 [0 - 0.028 AF]	H - Absence of amplification of the T allele
						g.105654710G>T	0.0008402 [0 - 0.0091 AF]	H - Absence of amplification of the T allele

**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.

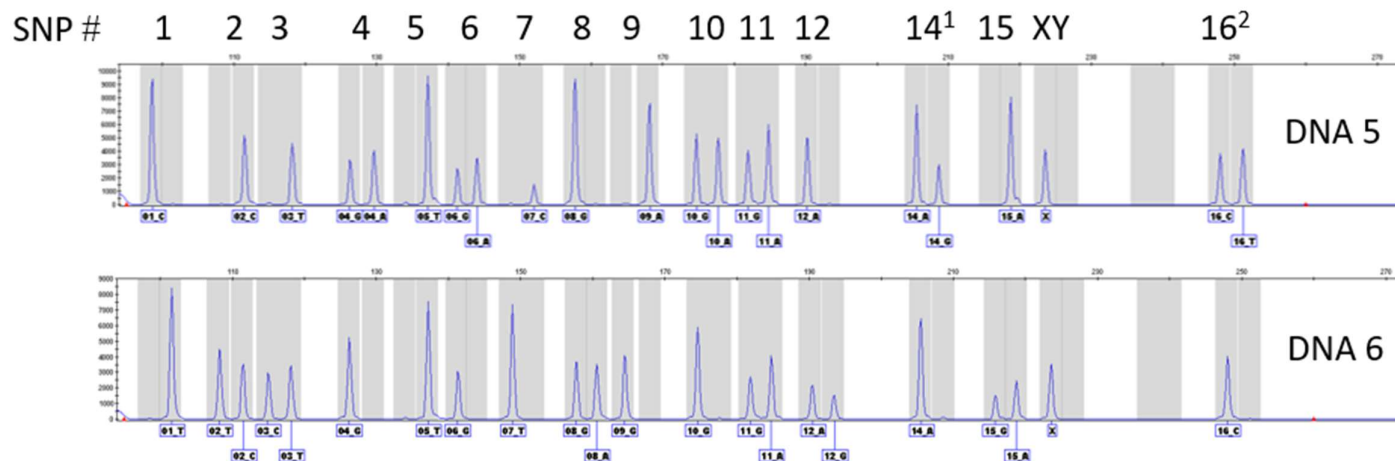
*** H: Interference on hybridization of one of the primers; T: interference on the size of the PCR product

IX. Example of profiles



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



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

X. Identification of symbols

Symbol	Description
	This symbol indicates the address of the manufacturer.
	Date of manufacture.
	Consult the instructions before use. A QR Code allows 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 so that the device can be formally identified.
	This symbol indicates the manufacturer's batch code so that the batch can be formally identified.
	This symbol indicates that the content is sufficient for “n” trials.