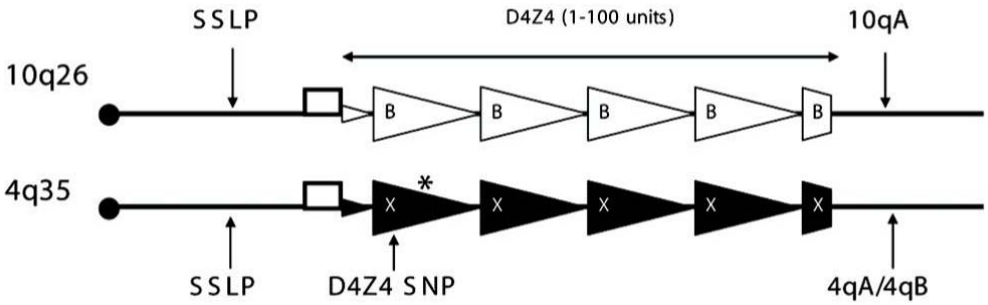
Genotyping of the SSLP

The simple sequence length polymorphism (SSLP) 3.5 kb proximal to D4Z4 is studied by PCR and the sequence is localized between positions 1532 and 1694 of AF117653 (see figure below).



2CAF (forward primer) 5′-GGTGGAGTTCTGGTTTCAGC-3′
2CAR (reverse primer) 5′-CCTGTGCTTCAGAGGCATTTG-3′
For fragment analysis, the forward primer is labelled with HEX.

Conditions for SSLP PCR

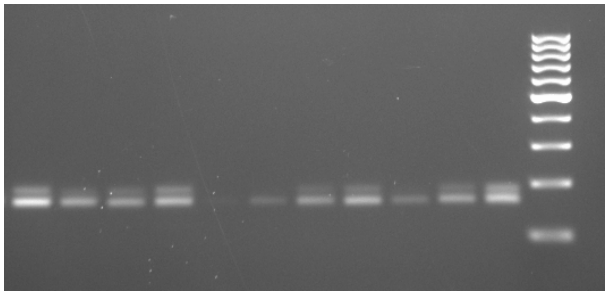
DNA (2,5 ng/uL)	2 uL
2CAF (10 uM)	0,4 uL
2CAR (10 uM)	0,4 uL
HF buffer	5 uL
dNTP (2 mM)	2,5 uL
Phusion enzyme	0,2 uL
water	14,5 uL
	25 uL

Conditions		
98C	3"	
98C	**15"**	
60C	**30"**	
72C	**15"**	32x
72C	30'	
15C	1'	

Dilution of PCR products for SSLP analysis:

As you can see in the protocol above, we only use a very small amount of input DNA in PCR. After the PCR we normally check the PCR-reaction on an agarose gel. Then we run 8 uL PCR reaction on gel, which yields about 25-50 ng PCR product. This product is then further diluted about 300 times for the fragment run.

For optimal determination of allele composition, peak height signals of a single allele peak should be between 1000 and 2000 AU (SoftGenetics GeneMarker). To optimize the fragment run result several dilutions of the input PCR product should be tested.



Electrophoresis picture of SSLP PCR products (on gel: 8 uL PCR product and 8 uL 100 bp GeneRuler molecular size marker).

For fragment analysis:

1. Dilute samples (directly from PCR mix, unpurified) to approx. 0,8-1 ng/uL.
2. Mix: 2 uL diluted PCR product + 10 uL ROX/formamide mix (2,5 uL ROX HD400 with 500 uL ionized formamide)

For the SSLP analysis you might also have problems with the double peaks.

- Input DNA concentration might have been too high.
- Try different PCR-enzymes, we obtained best results with the proofreading enzyme Phusion (with other enzymes [for instance amplitaq gold] we obtained double peaks).

Analysis of fragment run

Size differences in the SSLP fragments were determined with the use of an ABI Prism 3100 Genetic Analyzer.
SSLP haplotyping of these D4Z4 alleles requires some experience.

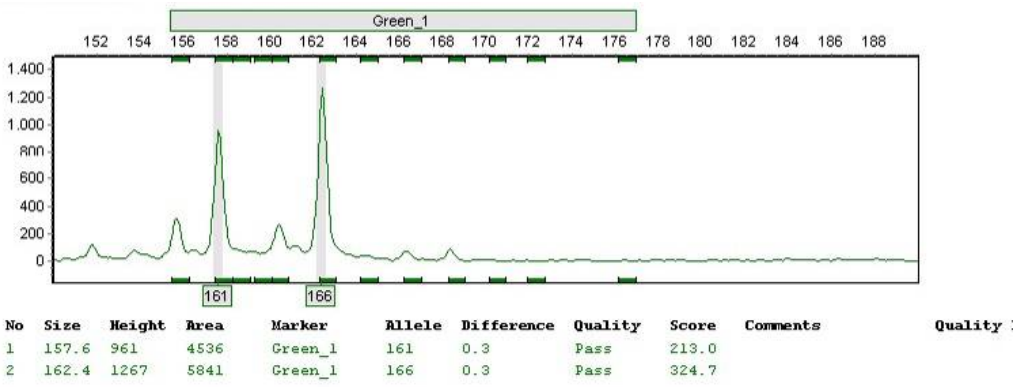
- All SSLP peaks appear 3 bp shorter than the actual PCR fragment, this is a running artifact (see examples below)
- Not all SSLP peaks appear with the same peak height on the fragment run-result (for example: 164>166>161>168>163>176,180).
- Furthermore some peaks appear with a stutter peak before the base-peak and others have a stutter peak behind the base peak.
- AJHG paper (2007) we show haplotypes that were found in individuals with a standard allele configuration, in our 2010 AJHG paper we show all haplotypes found in the European population.
- About 10% of the European individuals carry a 4-type repeat on 10q and 10% carry 10-type repeats on 4q. For these unusual alleles we observe other SSLP lengths (161 [10B161T], 176 [10A176T] and 180 [10A180T].
- Also other rare sizes (>170 bp) have been detected in our AJHG study.

Overview of the prevalence of haplotypes found in the European population (based on a study of 444 independent European controls, AJHG 2010)

Chromosome 4			Chromosome 10		
haplotype	**%**	**Genbank Acc. Nr.**	**haplotype**	**%**	**Genbank Acc. Nr.**
0 (deletion)	0.2	-	0 (deletion)	0.9	-
4A159	0.1	GU480773	10B161T	4.6	GU480777, GU480778
4A161	39.2	GU480774, GU480775	10A162	0.2	GU480801
4B161	0.7	GU480803	10A164	4.4	GU480799, GU480800
4B162	1.8	GU480791, GU480792	10A166	86.1	GU480802, GU480784, GU480785
4B163	32.9	GU480776	10A166H	0.6	GU480781, GU480782, GU480783
4A163	0.9	-	10A176T	2.5	GU480779
4A166	4.4	GU480786	10A180T	0.5	GU480780
4A166H	3,9	GU480787, GU480788, GU480789	other	0.2	-
4B166	1.0	-			
4B168	13.2	GU480793, GU480794			
4A168	0.3	GU480797, GU480798			
4A170	0.2	-			
4B170	0.5	GU480795			
4A172	0.2	-			
4B172	0.2	GU480796			
4B174	0.2	-			

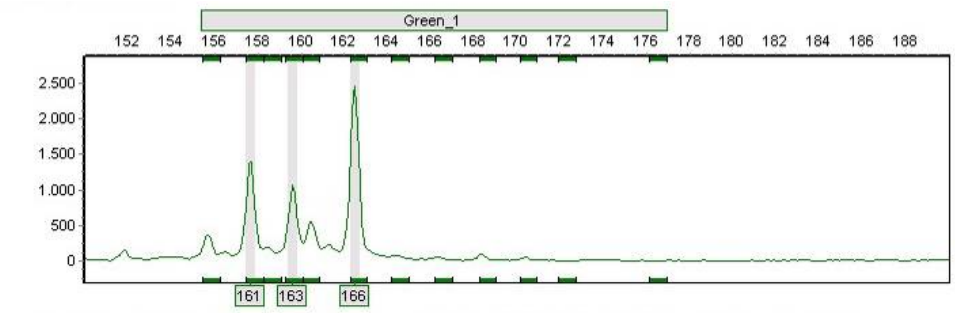
Below you will find some examples of haplotype combinations that we have observed in the European population (but many more combinations can be found). Between brackets is the prevalence of this genotype that can be expected, based on the incidence of the distinct haplotypes. Sometimes, different haplotype combinations give the same SSLP pattern (but in these cases one of the two is more likely).

	Chr. 4-1	Chr. 4-2	Chr. 10-1	Chr. 10-2	Incidence
Genotype	**4A161**	**4A161**	**10A166**	**10A166**	**11.2%**
Genotype	**4A161**	**4A166(H)**	**10A166**	**10B161T**	**0.1%**



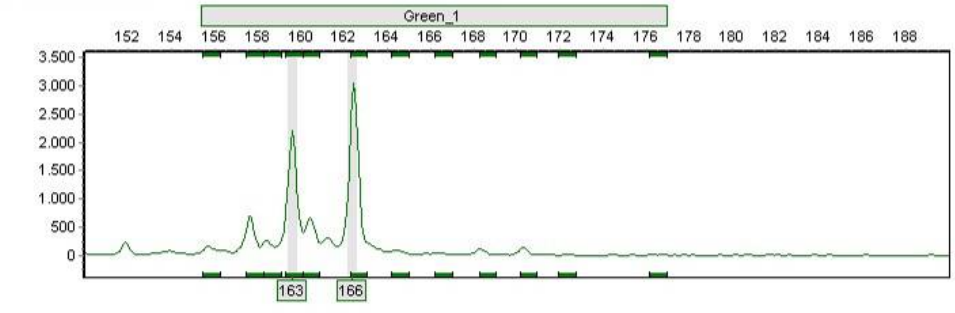
No	Size	Height	Area	Marker	Allele	Difference	Quality	Score	Comments	Quality
1	157.6	961	4536	Green_1	161	0.3	Pass	213.0		
2	162.4	1267	5841	Green_1	166	0.3	Pass	324.7		

	Chr. 4-1	Chr. 4-2	Chr. 10-1	Chr. 10-2	Incidence
Genotype	**4A161**	**4B163**	**10A166**	**10A166**	**9.5%**
Genotype	**4B163**	**4A166(H)**	**10A166**	**10B161T**	**0.1%**



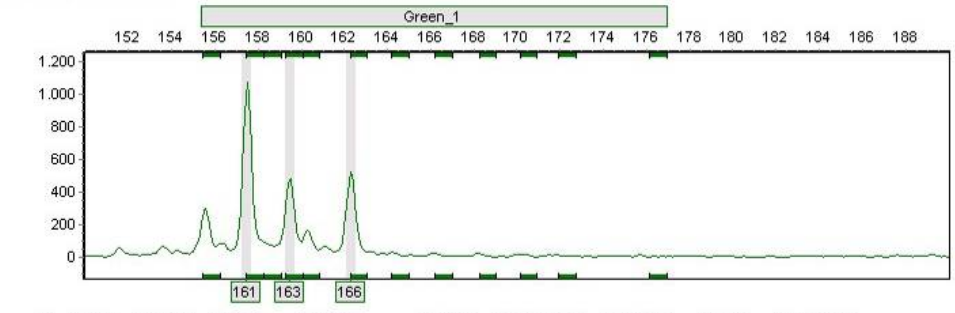
No	Size	Height	Area	Marker	Allele	Difference	Quality	Score	Comments	Quality
1	157.7	1394	6427	Green_1	161	0.2	Pass	253.6		
2	159.6	1058	5212	Green_1	163	0.1	Pass	157.4		
3	162.5	2455	11035	Green_1	166	0.2	Pass	500.0		

	Chr. 4-1	Chr. 4-2	Chr. 10-1	Chr. 10-2	Incidence
Genotype	**4B163**	**4B163**	**10A166**	**10A166**	**8.1%**



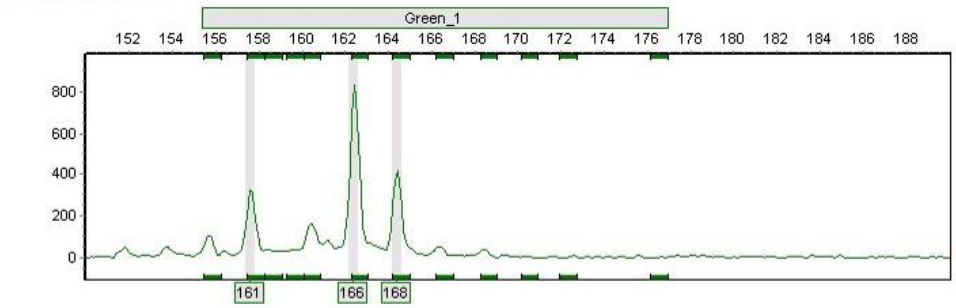
No	Size	Height	Area	Marker	Allele	Difference	Quality	Score	Comments	Quality
1	159.6	2216	10238	Green_1	163	0.1	Pass	325.0		
2	162.4	3048	13938	Green_1	166	0.3	Pass	500.0		

	Chr. 4-1	Chr. 4-2	Chr. 10-1	Chr. 10-2	Incidence
Genotype	**4B163**	**4B163**	**10A166**	**10B161T**	**6%**



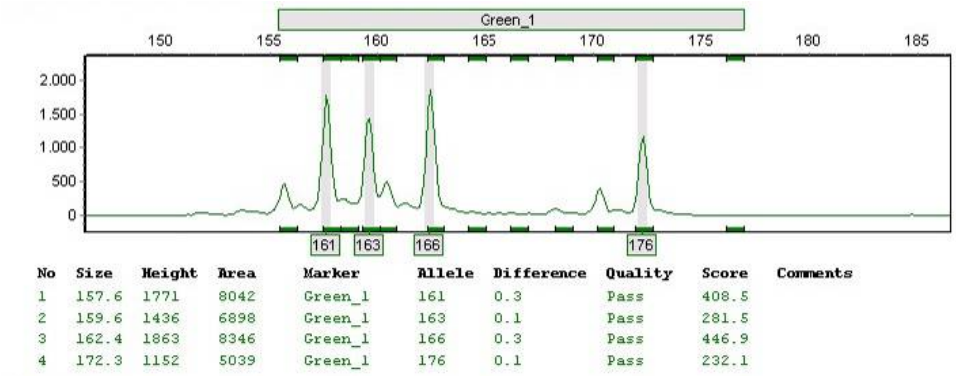
No	Size	Height	Area	Marker	Allele	Difference	Quality	Score	Comments	Quality
1	157.5	1076	4880	Green_1	161	0.4	Pass	229.3		
2	159.5	482	2408	Green_1	163	0.2	Pass	57.5		
3	162.3	529	2525	Green_1	166	0.4	Pass	73.9		

	Chr. 4-1	Chr. 4-2	Chr. 10-1	Chr. 10-2	Incidence
Genotype	**4A161**	**4B168**	**10A166**	**10A166**	**3.8%**
Genotype	**4A166(H)**	**4B168**	**10A166**	**10B161T**	**<0.1%**



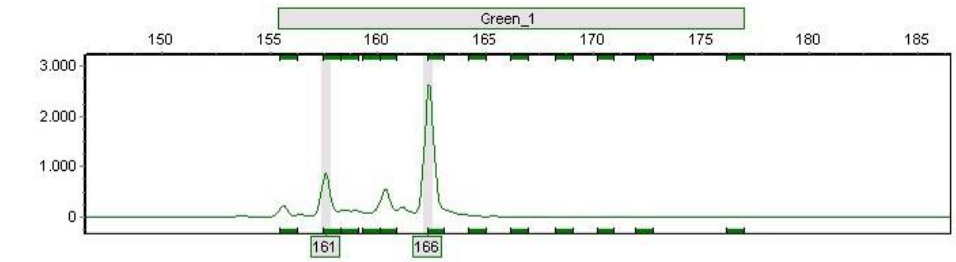
No	Size	Height	Area	Marker	Allele	Difference	Quality	Score	Comments	Quality
1	157.6	325	1533	Green_1	161	0.3	Pass	39.9		
2	162.4	832	3850	Green_1	166	0.3	Pass	176.3		
3	164.4	416	1945	Green_1	168	0.2	Pass	59.3		

	Chr. 4-1	Chr. 4-2	Chr. 10-1	Chr. 10-2	Incidence
Genotype	**4A161**	**4B163**	**10A166**	**10A176T**	**2.8%**



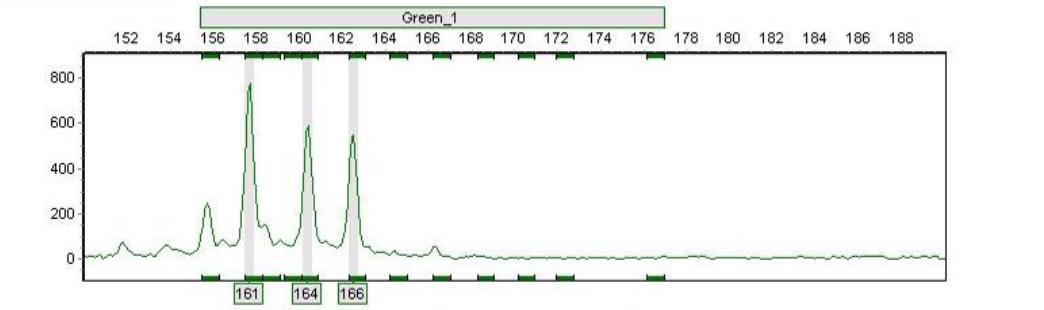
No	Size	Height	Area	Marker	Allele	Difference	Quality	Score	Comments	Quality
1	157.6	1771	8042	Green_1	161	0.3	Pass	408.5		
2	159.6	1436	6898	Green_1	163	0.1	Pass	281.5		
3	162.4	1863	8346	Green_1	166	0.3	Pass	446.9		
4	172.3	1152	5039	Green_1	176	0.1	Pass	232.1		

	Chr. 4-1	Chr. 4-2	Chr. 10-1	Chr. 10-2	Incidence
Genotype	**4A161**	**4A166(H)**	**10A166**	**10A166**	**2.3%**



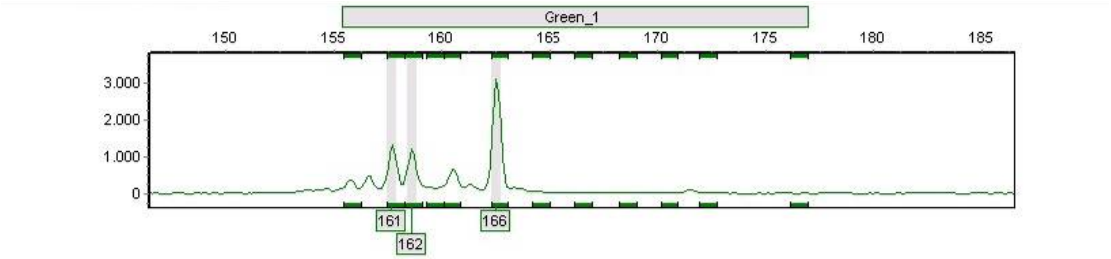
No	Size	Height	Area	Marker	Allele	Difference	Quality	Score	Comments	Quality
1	157.6	870	4036	Green_1	161	0.3	Pass	186.9		
2	162.3	2617	12548	Green_1	166	0.4	Pass	500.0		

	Chr. 4-1	Chr. 4-2	Chr. 10-1	Chr. 10-2	Incidence
Genotype	**4A161**	**4A161**	**10A164**	**10A166**	**0.6%**
Genotype	**4A161**	**4A166(H)**	**10A164**	**10B161T**	**<0.1%**



No	Size	Height	Area	Marker	Allele	Difference	Quality	Score	Comments	Quality
1	157.7	777	3748	Green_1	161	0.2	Pass	149.6		
2	160.4	589	3005	Green_1	164	0.1	Pass	92.7		
3	162.5	547	2612	Green_1	166	0.2	Pass	89.4		

	Chr. 4-1	Chr. 4-2	Chr. 10-1	Chr. 10-2	Incidence
Genotype	**4A161**	**4B162**	**10A166**	**10A166**	**0.5%**



No	Size	Height	Area	Marker	Allele	Difference	Quality	Score	Comments	Quality
1	157.7	1305	6050	Green_1	161	0.2	Pass	290.9		
2	158.6	1196	5828	Green_1	162	0.1	Pass	237.5		
3	162.5	3094	13365	Green_1	166	0.2	Pass	500.0		

End note:
You can not perform reliable FSHD genotyping (or diagnosis) only based on the SSLP length. When you consider all haplotypes that can be found, some quite frequently occur on 4q and 10q (like 161 and 166). Therefore, you have to combine the SSLP data with PFGE data for all D4Z4 repeats and 4qA/4qB data. The SSLP analysis certainly can assist to specify translocated alleles. Sometimes when you know the complete genotype (Southern and SSLP) of the parents the SSLP of the child can be sufficient to predict the alleles he/she inherited (but we always perform standard Southern analysis for diagnosis).