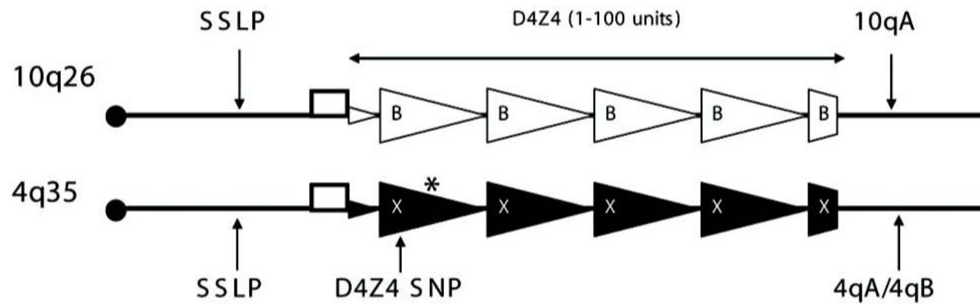
Genotyping of the SSLP

The SSLP proximal to D4Z4 were 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 was labeled with HEX.

Scheme for SSLP PCR

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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

Condities

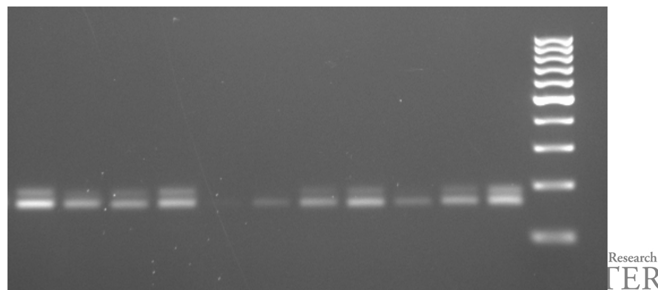
98C	3"	
98C	**15"**	
60C	**30"**	
72C	**15"**	32x
72C	30'	
15C	1'	

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 gives about 25-50 ng product.
This product is then further diluted about 300 times for the fragment run.

Maybe you better first try to use several dilutions of your PCR product in 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).

Enclosed is an electrophoresis picture of SSLP PCR products (primers used: 2CAF labeled + 2CAR).
On gel: 8 uL PCR product, 8 uL 100 bp ladder (GeneRuler).



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For fragment analysis:

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

Analysis of fragmentrun

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's shorter than the actual PCR fragment, this is a running artifact
- Not all SSLP peaks appear with the same peak height on the fragment run-result (164>166>161>168>163>176,180).
- Furthermore some peaks appear with a stutterpeak before the base-peak and others have a stutter peak behind the base peak.
- AJHG paper we shows haplotypes that were found in individuals with a standard allele configuration.
- However, many other rare haplotypes can be distinguished. For instance, about 10% of the European indiviaduals carry a 4-type repeat on 10q and 10% carry 10-type repeats on 4q. For these unusual alleles we sometimes observe other SSLP lengths (>170 bp).

Troubleshooting;

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

- Input DNA concentration might have been too high (or the input conc. for the fragment run are 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.

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End note:

You cannot perform reliable FSHD genotyping (or diagnosis) only based on the SSLP length. In our AJHG paper we have only discussed the standard haplotypes in individuals who did not have translocated alleles (no trisomic/monosomic individuals, only disomics were included). When you include trisomic and monosomic individuals you find much more haplotypes, and 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. Then, the SSLP can assist to specify translocated alleles, or to find false positive diagnosis). Sometimes when you know the complete genotype (with 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).