A modified mini-primer set for the mtDNA control region sequence analysis from highly degraded forensic remains

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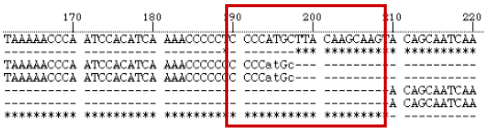
Mini-primer set strategy

- As highly degraded samples contain populations of intact DNA molecules that are severely restricted in size, **the mini-primer set amplification strategy** which **attempts to target and preferentially amplify authentic human mtDNA sequences with small PCR products** was suggested by Gabriel *et al*. and Edson *et al.* from AFDIL (Armed Forces DNA Identification Laboratory).

- These mini-primer sets recovered reliable sequences from **highly degraded skeletal remains** and showed a dramatic increase in amplification success rate when compared with those consisting of larger amplicons.

Limitation of the AFDIL mini-primer set

- A **sequence gap** can be caused on samples **with the HV1 length heteroplasmy**.



- AFDIL mini-primer can produce **reduced PCR yield or inhibited amplification** by some **frequent mutations**.

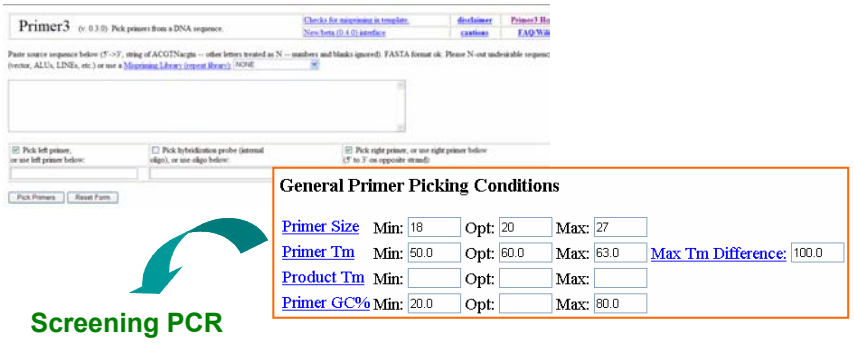
	n.p. 16140	n.p. 16209	n.p. 16304
AFDIL mini-primer	Mps1a (R16158)	Mps2a (F16190)	Mps2a (R16322)
3' end position	2nd nucleotide	1st nucleotide	2nd nucleotide
Caucasians	0.2%	**1.3%**	**8.6%**
Asians	**5.1%**	**3.2%**	**14.7%**
African Americans	0.3%	**6.3%**	0.9%

- Using a **different annealing temperature** for each primer pairs would make the mtDNA control region amplification often **cumbersome and time-consuming**.

Designing of a modified mini-primer set

- The modified mini-primer set was designed **with consideration of the HV1 length heteroplasmy, nucleotide variability within the control region and primer T_m** for the simultaneous amplification.

Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)



Screening PCR

➡ The final set of primers with high amplification efficiency, relatively short primer length, and high Tm.

Modified mini-primer set for the mitochondrial DNA control region sequence analysis

Region	Amplicon	Primer sequence (5′ → 3′)		Amplicon size
HV1	M11	F15989	CCC AAA GCT AAG ATT CTA AT	165bp
		R16153	CAG GTG GTC AAG TAT TTA TGG	
	M12	F16097	TAC ATT ACT GCC AGC CAC CA	137bp
		R16233	TGA TAG TTG AAG GTT GAT TGC TGT	
	M13	F16159	CAT AAA AAC CCA ATC CAC AT	146bp
		R16304	ACT GTT AAG GGT GGG TAG GT	
	M14	F16247	ACT CCA AAG CCA CCC CTC A	164bp
		R16410	GAG GAT GGT GGT CAA GGG AC	
HV2	M21	F015	CAC CCT ATT AAC CAC TCA CG	173bp
		R187	CGC CTG TAA TAT TGA ACG TA	
	M22	F120	CGC AGT ATC TGT CTT TGA TTC C	166bp
		R285	GTT ATG ATG TCT GTG TGG AA	
	M23	F220	TGC TTG TAG GAC ATA ATA AT	170bp
		R389	CTG GTT AGG CTG GTG TTA GG	

[a]Primers which were newly designed were indicated in green.

[b]To facilitate the PCR amplification, R16233 primer sequence has the nucleotide A instead of nucleotide G at n.p. The nucleotide which is complementary to the 16223T is underlined.

PCR amplification efficiency test

➥ **To evaluate the final modified set of primers, test DNA sample were simultaneously amplified with the modified mini-primer set and the AFDIL mini-primer set.**

❖ DNA samples
- **DNA from blood samples**
- **Samples from 55 year-old skeletal remains**

❖ PCR mixture
- **Total 25.0 μl PCR reaction: 2 μl of DNA, 2.5 μl of Gold ST*R buffer**
- **2.5 U AmpliTaq Gold polymerase (1.0 U for blood sample) and primer**

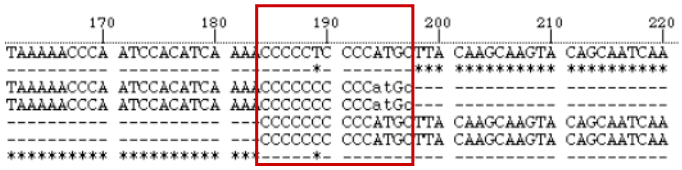
❖ Thermal Cycling

95℃ for 11 min
95℃ for 20 sec
50℃ for 20 sec
72℃ for 30 sec
72℃ for 7 min

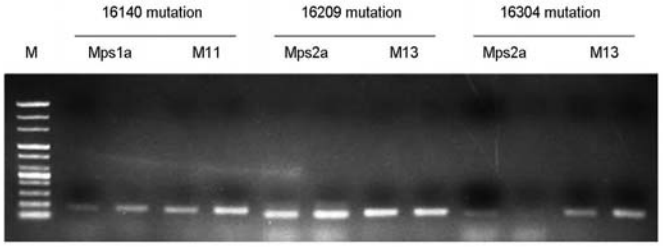
X 42 cycles (old skeletal remains)
Blood sample : annealing Tm - 56℃, 35 cycles

Sequence alignment and frequent mutation

- The modified mini-primer set enables the entire HV1 region sequence to be obtained without missing a nucleotide on samples with the 16189 mutation,



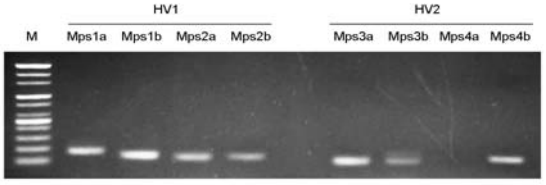
- The modified mini-primer set was less affected by frequent mutations than those of the AFDIL.



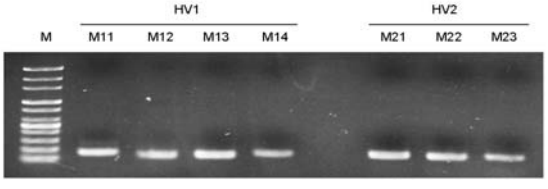
PCR efficiency comparison on highly degraded skeletal remains

- The modified mini-primer set worked on highly degraded samples with much more efficiency than the AFDIL mini-primer set

A. AFDIL mini-primer

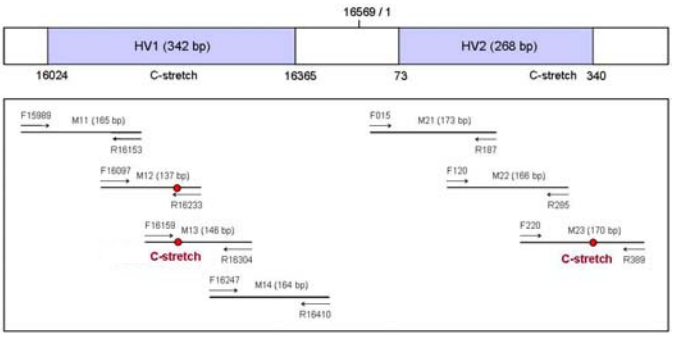


B. Modified mini-primer



- Equal volumes of each PCR product were analyzed on an agarose gel, and Mps4b and M23 were amplified with the same primer pair in the two mini-primer sets.

The modified mini-primer set



- The modified mini-primer set is composed of **four and three PCR amplicons**.
- The forward primer of M13 was designed to be **avoid the possible gap** on samples **with the HV1 length heteroplasmy**.
- Using the FBI mtDNA population database, **the first to the third nucleotide from the 3' end of each primer** were located at the nucleotide position with a **low mutation frequency** in the Caucasians, Asians, and Africans.

Alternative primers suggested

- As **high sequence variability over the entire control region** hindered the design of primers which are universally applicable to every population, several alternative primers are suggested for use in certain populations.

  - **In Africans**

    n.p. 16265 mutation frequency: **6.5%**

    M14 - F16247 → F16255 (5'-GCC ACC CCT CAC CCA CTA G)

    M13 - R16304 → R16322 (5'-TGG CTT TAT GTA CTA TGT AC)

  - **In Caucasians**

    n.p. 239 mutation frequency: **1.9%**

    M23 – F220 → F220* (5'-TGC TTG TAG GAC ATA ATA AT**A ACA A**)

Conclusion

- To facilitate **mitochondrial DNA analysis on highly degraded skeletal remains**, a modified mini-primer set was designed to **overcome the limitations of the AFDIL** (Armed Forces DNA Identification Laboratory) **mini-primer set**.
- The modified mini-primer set is **less affected by length heteroplsmy**, **nucleotide variability** and **PCR amplification** conditions than the AFDIL mini-primer set.
- As this modified mini-primer set was used successfully on 55-year-old skeletal remains with high efficiency, it will be a useful tool for **mtDNA control region sequence** analysis from **highly degraded forensic samples**.

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