APPENDIX A – OLIGONUCLEOTIDE PRIMER SEQUENCES[1]

Region	Primer	Nucleotide (base) Sequence	Size (no. of bases)
HVI	A1	5'- CAC CAT TAG CAC CCA AAG CT -3'	20
	A4	5'- CCC CAT GCT TAC AAG CAA GT -3'	20
	B1	5'- GAG GAT GGT GGT CAA GGG AC -3'	20
	B4	5'- TTT GAT GTG GAT TGG GTT T -3'	19
	HVIF	5'- CTC CAC CAT TAG CAC CCA A -3'	19
	HVIR	5'- ATT TCA CGG AGG ATG GTC -3'	18
HVII	C1	5'- CTC ACG GGA GCT CTC CAT GC -3'	20
	C2	5'- TTA TTT ATC GCA CCT ACG TTC AAT -3'	24
	D1	5'- CTG TTA AAA GTG CAT ACC GCC A -3'	22
	D2	5'- GGG GTT TGG TGG AAA TTT TTT G -3'	22
	HVIIF	5'- CAC CCT ATT AAC CAC TCA CG -3'	20
	HVIIR	5'- CTG TTA AAA GTG CAT ACC GC -3'	20

[1] Nucleotide sequences for primers A1, A4, B1, B4, C1, C2, D1, and D2 are from the FBI Laboratory DNA Analysis Unit II Mitochondrial DNA Analysis Protocol (mtDNA Protocol Manual, DNA Amplification - Rev. 8, Issue Date 02/01/05 for primers A1, B1, C1, C2, D1, and D2; mtDNA Protocol Manual, Cycle Sequencing - Rev. 8, Issue Date 09/10/04 for primers A4 and B4). The primer sequences in the FBI mtDNA Protocol Manual are based on those described in the following:

Wilson MR, DiZinno JA, Polanskey D, Replogle J, Budowle, B. Validation of mitochondrial DNA sequencing for forensic casework analysis. Int J of Leg Med 1995; 108:68-74.

Wilson MR, Polanskey D, Butler J, DiZinno JA, Replogle J, Budowle B. Extraction, PCR amplification, and sequencing of mitochondrial DNA from human hair shafts, BioTechniques 1995; 18(4):662-669.

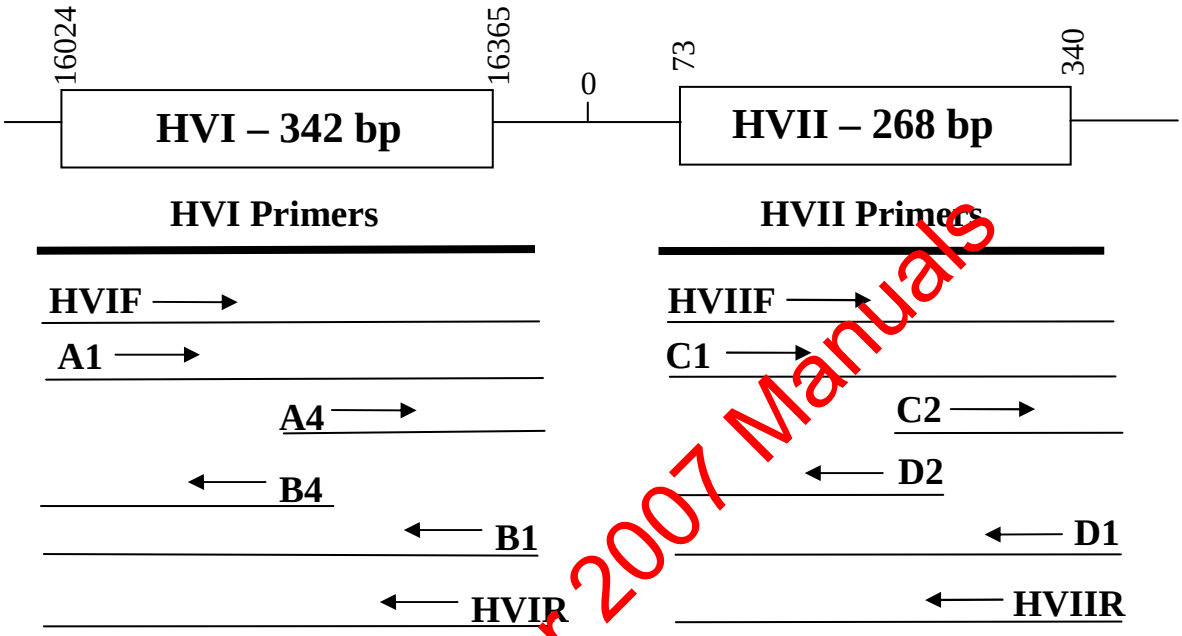
Nucleotide sequences for oligonucleotide primers HVIF, HVIR, HVIIF, HVIIR are from the product insert for the LINEAR ARRAY Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit that is available from Roche Applied Sciences (Cat. No. 03-527-867-001; product information is available at www.roche-applied-science.com). The primer sequences in the typing kit are based on those described in:

Gabriel MN, Calloway CD, Reynolds RL, Primorac D. Identification of human remains by immobilized sequence-specific oligonucleotide probe analysis of mtDNA hepervariable regions I and II. Croat Med J 2003; 44:293-298.

Kline MC, Vallone PM, Redman JW, Duewer DL, Calloway CD, Butler JM. Mitochondrial DNA typing screens with control region and coding region SNPs. J Forensic Sci 2005; 50:377-385.

APPENDIX B – MITOCHONDRIAL DNA PRIMER LOCATIONS[4]

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 1



HVI (16024 - 16365) = 342 bp		HVII (73 - 340) = 268 bp	
Primer	**Position[1]**	**Primer**	**Position[1]**
HVIF	15975	HVIIF	15
A1	15978	C1	29
A4[2]	16190	C2	154
B4[2]	16182	D2[3]	306
B1	16410	D1	429
HVIR	16418	HVIIR	429

[1] Nucleotide position is defined as the first base at the 5' end of the primer.
[2] Primers A4 and B4 are used to resolve C-stretch length polymorphisms in HVI.
[3] Primer D2 is used when necessary to resolve the reverse strand sequence when C-stretch polymorphism is present in HVII.
[4] The above diagrams are not to scale. All primer positions are relative to the table below. All arrows indicate the directions (forward or reverse) that the primer amplifies along the hypervariable region.

APPENDIX C – REVISED CAMBRIDGE REFERENCE SEQUENCE[1]

Hypervariable Region I (HVI)

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16024- TTCTTTCATG GGGAAGCAGA TTTGGGTACC ACCCAAGTAT
16064- TGACTCACCC ATCAACAACC GCTATGTATT TCGTACATTA
16104- CTGCCAGCCA CCATGAATAT TGTACGGTAC CATAAATACT
16144- TGACCACCTG TAGTACATAA AAACCCAATC CACATCAAAA
16184- CCCCCTCCCC ATGCTTACAA GCAAGTACAG CAATCAACCC
16224- TCAACTATCA CACATCAACT GCAACTCCAA AGCCACCCCT
16264- CACCCACTAG GATACCAACA AACCTACCCA CCCTTAACAG
16304- TACATAGTAC ATAAAGCCAT TTACCGTACA TAGCACATTA
16344- CAGTCAAATC CCTTCTCGTC CC -16365(end)
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Hypervariable Region II (HVII)

```
  73- ATGCACGCGA TAGCATTGCG AGACGCTGGA GCCGGAGCAC
 113- CCTATGTCGC AGTATCTGTC TTTGATTCCT GCCTCATCCT
 153- ATTATTTATC GCACCTACGT TCAATATTAC AGGCGAACAT
 193- ACTTACTAAA GTGTGTTAAT TAATTAATGC TTGTAGGACA
 233- TAATAATAAC AATTGAATGT CTGCACAGCC ACTTTCCACA
 273- CAGACATCAT AACAAAAAAT TTCCACCAAA CCCCCCCTCC
 313- CCCGCTTCTG GCCACAGCAC TTAAACAC- 340(end)
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[1] Human Mitochondrial DNA Revised Cambridge Reference Sequence,
from http://www.gen.emory.edu/MITOMAP/mitoseq.html
LOCUS HUMMTCG 16568 bp ds-DNA Circular PRI 27-Sept-2001
DEFINITION- Human mitochondrion, complete genome.
ACCESSION- This sequence is a modified version of the 2001 Revised Cambridge Reference Sequence (GenBank #NC_001807, a derivation of #J01415)
SOURCE Human placenta mitochondrial DNA
ORGANISM Mitochondrion Homo sapiens- Eukaryota; Animalia; Chordata; Vertebrata; Mammalia; Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae.
REFRENCES

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome Nature 1981; 290: 457-465. MEDLINE- 81173052 PUBMED-7219534

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat. Genet. 1999; 23 (2): 147. MEDLINE-99438386 PUBMED- 10508508

Example:

Sample	# of Gels	# of LA's*	LA* input vol. (ul)	Misc. vol. (ul)	Gel Value/4 ul	ExoSap-It (ul)	Template (ul)	Comment	Water (ul)
Amp. Neg	1	0	0	0	0	9.2	3.0	Control	7.8
Sample	1	1	5.4	1	56	7.9	4.3	1/10 dil.	6.5

* LA = linear array analysis, currently only used for quality control purposes

Calculations for Amplification Negative:

1. The amplification negative was run only once on a gel at the usual volume of 4 ul. The gel value was zero for that sample, and a linear array was not run. Therefore, the analyst enters 1 into the "# of Gels" field and 0 each into the "# of LA's", "LA input vol.", "Misc vol.", and "Gel value" fields.
2. The total remaining reaction volume is calculated by the spreadsheet to be 46 ul (50 ul starting volume minus 4 ul used for gel analysis).
3. The amount of ExoSAP-IT required is calculated by the spreadsheet according to the following guideline: 1 ul of ExoSAP-IT for every 5 ul of amplified product. Thus, the current reaction volume is divided by 5 (46/5) and the spreadsheet enters 9.2 into the "ExoSAP-IT" field.
4. The amount of template to add to the cycle sequencing reaction is calculated by the spreadsheet. In this case, a gel value of 0 instructs the program to enter the maximum volume amount of 3 ul into the template field. The spreadsheet also enters "Control" into the comments field based on the 0 gel value.
5. Finally, the amount of water is calculated based on the previously calculated sample volume to make the total volume quantity sufficient at 10.8 ul. This DNA/water mixture is now ready to be added to the cycle sequencing reaction.

Calculations for Sample:

1. In this example, in addition to the first gel another gel was run using only 1 ul of amplified sample. This was done since the result of the first gel was off-scale relative to the upper molecular mass gel standard. Also, 5.4 ul of the sample was used for Linear Array analysis. The user inputs 1 into the "# of Gels" field since only 1 gel was run at the usual volume of 4 ul. The user also inputs 1 into the "# of LA's" field along with the volume of 5.4 (ul) used for the Linear Array analysis into the "LA input vol." field. To account for the additional 1 ul that was run on the second gel, this volume is inputted into the "Misc. Vol." field. Finally, the user inputs the gel value into the "Gel Value" field. This field is based on the amount of sample DNA in a volume of 4 ul. In this case, the gel rerun yielded a value of 14 ng/ul. This value is then corrected by the user and multiplied by 4 to yield the final value of 56.
2. The total reaction volume is calculated by the spreadsheet to be 39.6 ul, which is equal to the starting volume minus 4 ul for the first gel, 5.4 ul used for the Linear Array, and 1 ul that was used for the second gel (50 - 4 - 5.4 - 1 = 39.6).
3. The amount of ExoSAP-IT required is then determined as before. The spreadsheet calculates that the reaction requires 7.9 ul of ExoSAP-IT (39.6/5).
4. In calculating the amount of template required for the cycle sequencing reaction, the spreadsheet first determines the new concentration of the DNA sample after addition of ExoSAP-IT by the dividing the original concentration [(56 ng/4 ul)(39.6 ul)] by the new volume (47.5 ul). The new concentration (11.67 ng/ul) is then used to calculate the volume of sample needed to equal 5 ng of sample DNA [(5 ng)/(11.67 ng/ul) = 0.428 ul]. If the final volume is less than 1 ul, the spreadsheet will indicate that a dilution is necessary in the "Comment" filed. In this example, the spreadsheet calculation indicates that 4.3 ul of a 1/10 sample dilution is required.
5. As before, the spreadsheet indicates the amount of water necessary to yield a total of 10.8 ul of sample volume for the next step of cycle sequencing.