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A novel polymerase chain reaction (PCR) based assay for authentication of cell lines or tissues from human, pig and chicken origin

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ABSTRACT

A polymerase chain reaction based assay was developed for authentication of cell lines or tissues from human, pig and chicken origin. Specificity was achieved by species specific primer design targeting the mitochondrial *D-loop* sequence. Amplicon sizes were 114 bp, 169 bp and 645-648 bp for chicken, human and pig derived cell lines, respectively. Primers were tested for species specificity and non-specificity between haplogroups of the same organisms using BLAST tool and subsequently for cross amplification DNA extracted from human, chicken and pig venous blood as a positive control. Primers were also amplifying specific products in DNA extracted from individual cell line in both functional cell models and intentionally mixed cell lines consisting functional cell models. The PCR assay developed in this study represents a low-cost species specific end-point PCR based assay of the mitochondrial D-loop for the authentication of the cell line origin.

Key words: Polymerase chain reaction, mitochondrial *D-loop*, functional cell model, cell line authentication, human, pig, chicken

Introduction

Cell line models are offering a suitable alternative for *in vivo* animal testing with many advantages like simplicity, inter-laboratory repeatability and large scale testing capacity. Most of the available cell lines are deriving from tumorigenic origin therefore their reliability and quality of the results obtained are questionable. A good alternative to cancer derived cell lines are normal cell lines isolated from healthy dissected tissue, where tissue characteristics are generally better preserved (Langerholc et al., 2011). Cencic and colleagues have developed several functional small intestinal cell models of human and animal origin in the frame of PathogenCombat project (EU FP6 programme) (Cencic & Langerholc, 2010; Langerholc et al., 2011). As it has been shown in the past, many cell lines are not from the origin stated, due to contamination with other cell lines (Lavappa et al., 1976).

Species identification using the PCR amplification of mitochondrial *D-loop* sequence is gaining importance recently (Mane et al., 2009). The techniques for identification of meat and animal species were developed using species specific polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) of nuclear or mitochondrial markers (Koh et al., 1998; Partis et al., 2000; Verkaar et al., 2002; Saez, 2004; Ilhak & Arslan, 2007; Rastogi et al., 2007). Mitochondrial markers for species identification were designed using *12s rRNA gene* sequence or *D-loop* sequence (Girish, 2004; Girish et al., 2005; Fajardo et al., 2007; Fajardo et al., 2008;). Identification of origin of species by PCR using species-specific markers of *D-loop* origin of mitochondria is relatively quick, precise, sensitive and economical as compared to other PCR based assays (Haunshi et al., 2009).

The present study was carried out to develop a low-cost species specific end-point PCR based assay of the mitochondrial *D-loop* for the authentication of the cell line origin.

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Materials and Methods

Collection of blood samples and DNA extraction from white blood cells

Twelve milliliters of blood was aseptically collected into EDTA tubes (Greiner Bio-one, Frickenhausen, Germany) from cubital veins in humans at the University hospital, jugular or wing veins in chickens and from anterior vena cava in pigs at the University farm. Initially, the venous blood was separated to plasma, erythrocytes and white blood cells using the Ficoll Paque PLUS (GE Healthcare life sciences, Uppsala, Sweden) according to the manufacturer's instructions. The white blood cells were transferred into new sterile centrifuge tubes and stored at -70°C for DNA extraction. DNA was isolated from the white blood cells using the TRI reagent (Sigma, Steinheim, Germany) according to the manufacturer's instructions. Extracted DNA from venous blood was used as a positive control in comparison with DNA extracted from cell lines during the present study.

Functional cell models and DNA extraction from the cell lines

Functional cell models were built using non-carcinogenic cell lines (Table 1) from human, pig and chicken origin. Cells were seeded at the concentration $5x10^5$ into each insert well with 0.4 μm microporous membrane (Costar, Corning, New York) on a twelve well plate, one cell line per one 12 well plate (Costar, Corning, New York) and grown in DMEM advanced medium (Life technologies, Carlsbad, California) supplied with 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 2 mmol L-Glutamine (Life technologies, Carlsbad, USA) and 5% foetal bovine serum (Life technologies, Carlsbad, USA) in humidified 5% CO_2 atmosphere at 37°C. When the cell monolayer was formed and the transepithelial resistances reached approximately 800 Ω, cells were detached with 0.5 mL of 0.25% trypsine-EDTA solution, collected to a centrifuge tube, additionally resuspended in DMEM advanced medium and centrifuged at 850 rpm for 5 minutes. After centrifugation the supernatant was removed, the cell sediment was resuspended in sterile PBS buffer and centrifuged again at 1400 rpm for 15 minutes. DNA was isolated from the cell sediment with the same procedure as described before.

Checking quality and purity of the extracted DNA

The quality of isolated DNA was checked by agarose gel electrophoresis using 2% agarose strength. The concentration of extracted DNA was measured with optical density readings at 260 nm and purity was calculated by taking the ratio of 260 nm and 280 nm using the photometer (Eppendorf, Hamburg, Germany). Obtained DNA concentration was diluted with sterilized double distilled H_2O to final concentration 50 ng/μL.

Table 1. *Cell lines used in functional cell models in present study*.

Name and reference	Tissue	Species
UH – primary cell line	Human uretra	*Homo sapiens*
BH – primary cell line	Human bladder	*Homo sapiens*
PSI (Cencic, 2008; Gradisnik et al., 2006)	Pig small intestine	*Sus scrofa*
CLAB (Cencic, 2008; Gradisnik et al., 2006)	Pig small intestine	*Sus scrofa*
PTC – primary cell line	Chicken small intestine	*Gallus gallus*
C2 – primary cell line	Chicken small intestine	*Gallus gallus*

Retrieving sequence and primer design

Sequences for primer design were retrieved from PubMed Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) (Table 2). Primers were designed specific for species but non-specific for haplogroups in species.

Table 2. *Retrieved mitochondrial D-loop sequences.*

Homo sapiens	*Homo sapiens*	*Sus scrofa*	*Gallus gallus*
HQ700378.2	JF703252.1	GU147934.1	GU261719.1
HM044856.1	HM153530.1	DQ518915.2	GU261718.1
HM044854.1	JF904935.1	AF034253.1	GU261716.1
HM804484.1	JF811749.1	NC_000845.1	GU261700.1
HM804483.1	JF812599.1	D16483.1	GU261679.1
HM804486.1	JF812166.1	DQ518915.2	GU261676.1
JF905568.1	HQ436101.1	EF545589.1	AY235571.1
JF825889.1	JF813785.1	EF545590.1	
JF831421.1	JF819714.1	EF545586.1	
JF903810.1	FJ445408.2	EF545577.1	
JF833040.1	HM238208.1	DQ207754.1	
JF833037.1	JF487827.1	AY574046.1	
JF828090.1	JF499899.1	AY574045.1	
JF830105.1	JF502419.1	AY337045.1	
JF893456.1	JF509360.1	GQ220328.1	
JF829690.1	JF433953.1	DQ466081.2	
JF903930.1	JF436855.1	EU333163.1	
JF903929.1	JF298212.1	EF375877.3	
JF303729.1			
HQ907958.1			

Retrieved sequences were aligned using a web sequence aligning tool ClustalW2

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(http://www.ebi.ac.uk/Tools/msa/clustalw2/) for targeting the specific sequences between species and non-specific sequences between different haplogroups in same species. Primers were designed by hand and checked using a primer analyzing web tool IDT oligo analyzer (http://eu.idtDNA.com/analyzer/Applications/OligoAnalyzer) (Table 3), custom synthesis of primers was obtained from Sigma, Steinheim, Germany.

Table 3. *Species specific primers designed for the present study*.

Species	Primer name	Primer sequence 5' – 3'	Amplicon size (bp)
Homo sapiens	HS_MTdl_FW	ATACTGCGACATAGGGTGCT	169
	HS_MTdl_RV	CTAAATAGCCCACACGTTCC	
Sus scrofa	SS_MTdl_FW	ACCAAAACAAGCATTCCAT	645-648
	SS_MTdl_RV	GGATCATGAGTTCCATGAAG	
Gallus gallus	GG_MTdl_FW	TACTTCATGACCAGTCTCAGG	114
	GG_MTdl_RV	AGTTCAGGAGTTATGCATGG	

Optimization of PCR procedures

PCR reactions were carried out in a volume of 20 µL reaction mixture containing 4 µL of DNA and 16 µL of PCR mix. PCR mix was containing 8.3 µL of sterilized double distilled H_2O, 10x PCR Buffer (Sigma, Steinheim, Germany), $MgCl_2$ (Sigma, Steinheim, Germany), 0.1 µL TAQ polymerase 5 U/µL (Sigma, Steinheim, Germany) and 2 µL of each forward and reverse primers with concentration 2.5 µmol/L. Optimized thermal profile of PCR assay is given in Table 4.

Table 4. *Optimized thermal profile of the PCR assay*.

Initial denaturation	94°C	5 minutes
Denaturation	94°C	30 seconds
Annealing	55°C	30 seconds
Elongation	72°C	30 seconds
Final elongation	72°C	7 minutes
Number of cycles	35 cycles	

PCR amplification was carried out in programmable thermo cycler (Biometra, Goettingen, Germany). After amplification PCR products were analyzed on 2% agarose gel in 1x TBE buffer containing ethidium bromide and visualized under UV light.

Results

Species specific primers designed in present study were initially submitted to nucleotide basic local alignment tool – nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to check the specificity between species and non-specificity between haplogroups of the same species (Table 5).

Table 5. *BLAST alignment search results*.

Primer pair	Number of specific sequences with 100% match
HS_MTdl	4591 sequences
SS_MTdl	998 sequences
GG_MTdl	1020 sequences

Results retrieved indicated unique primer design according to human, pig and chicken origin. Designed primers were also initially tested with positive DNA control extracted from white blood cells and were successfully amplifying unique fragments for human, pig and chicken (Figure 1).

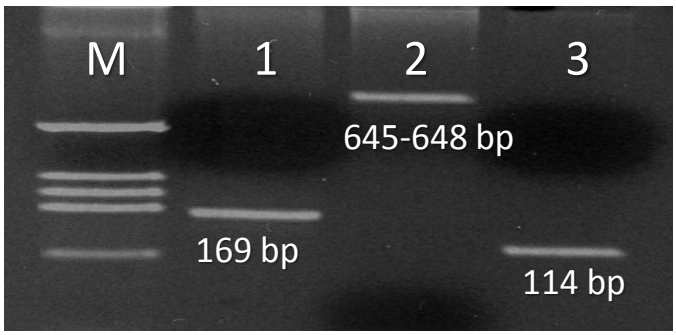

Figure 1. *Lane 1 is human DNA extracted from white blood cells amplified with HS_MTdl primer pair, lane 2 is pig DNA extracted from white blood cells amplified with SS_MTdl primer pair, lane 3 is chicken DNA extracted from white blood cells amplified with GG_MTdl primer pair and lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments*.

Species specific primers were further validated for cross amplification between human, pig and chicken DNA. From Figure 2 can be seen that primers designed for human species (HS_MTdl) successfully amplified the 169 bp fragment in human DNA but no amplification of products occurred in pig and chicken DNA. Similarly, DNA samples from pig were successfully amplified with pig primers (SS_MTdl) generating a 645-648 bp fragment but again, no amplification of products occurred in human and chicken DNA (Figure 3).

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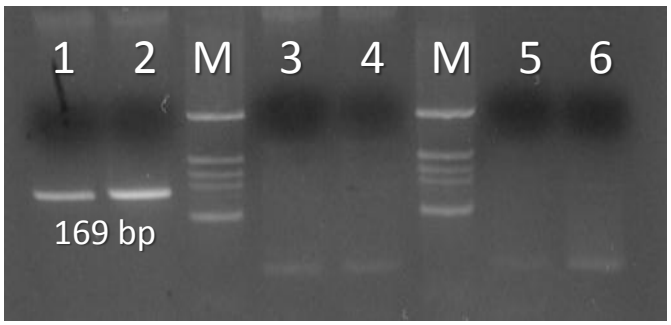

Figure 2. *HS_MTdl primer pair cross testing with human, pig and chicken DNA extracted from white blood cells. Lanes 1 and 2 are human DNA, lanes 3 and 4 are pig DNA, lanes 5 and 6 are chicken DNA and lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments.*

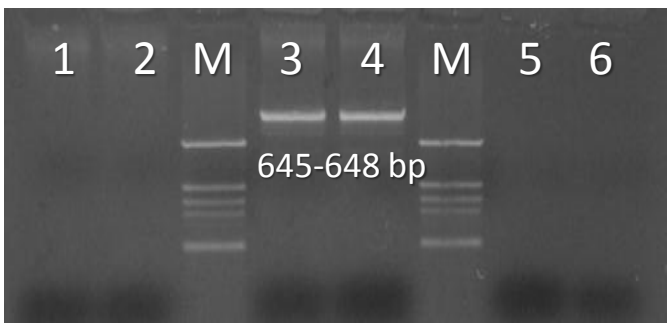

Figure 3. *SS_MTdl primer pair cross testing with human, pig and chicken DNA extracted from white blood cells. Lanes 1 and 2 are human DNA, lanes 3 and 4 are pig DNA, lanes 5 and 6 are chicken DNA and lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments.*

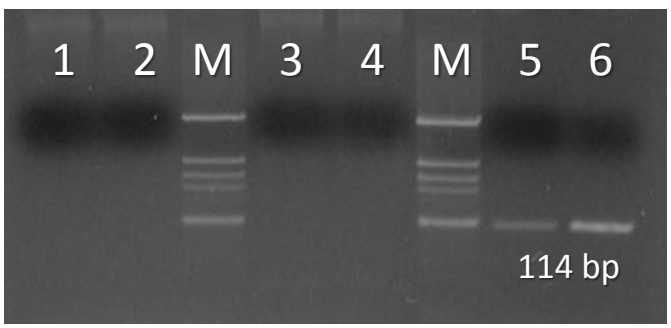

Figure 4. *GG_MTdl primer pair cross testing with human, pig and chicken DNA extracted from white blood cells. Lanes 1 and 2 are human DNA, lanes 3 and 4 are pig DNA, lanes 5 and 6 are chicken DNA and lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments.*

Primers designed for chicken species (GG_MTdl) amplified a 114 bp fragment in chicken DNA without showing any amplification in human or pig DNA (Figure 4).

Finally, the experiment for cell line testing was set up. We used human UH, BH cell lines, pig CLAB, PSI cell lines and chicken PTC, C2 cell lines and built nine different functional cell models where first six models contained cell lines stated above and last three contained mixed cell lines. The first mixed cell model was consisted of BH and PSI cells lines, the second UH and PTC cell line and the third model was consisted of CLAB and C2 cell lines.

Primers designed for human species (HS_MTdl) successfully amplified the 169 bp fragment in DNA extracted from cell lines in human functional cell models but no amplification of DNA occurred in DNA extracted from pig and chicken cell lines in functional cell models (Figure 5).

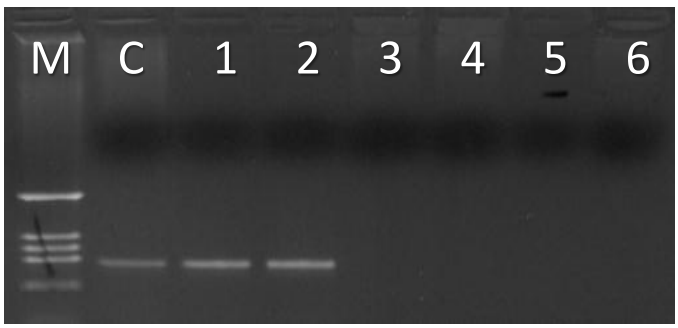

Figure 5. *Amplification of DNA extracted from functional cell models with HS_MTdl primer pair. Lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments, lane C is positive control (human DNA extracted from venous blood). Lane 1 is DNA extracted from functional cell model with UH cell line, lane 2 is DNA extracted from functional cell model with BH cell line, lane 3 is DNA extracted from functional cell model with PSI cell line, lane 4 is DNA extracted from functional cell model with CLAB cell line, lane 5 is DNA extracted from functional cell model with C2 cell line and lane 6 is DNA extracted from functional cell model with PTC cell line.*

Similarly, DNA extracted from cell lines in pig functional cell models was successfully amplified with pig primers (SS_MTdl) generating a 645-648 bp fragment but again, no amplification of products occurred in DNA extracted from cell lines in human and chicken functional cell models (Figure 6).

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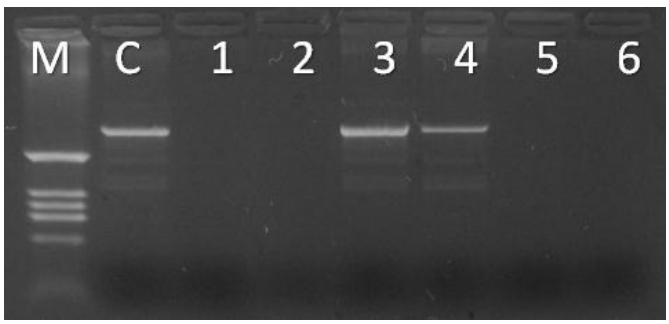

Figure 6. *Amplification of DNA extracted from functional cell models with SS_MTdl primer pair. Lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments, lane C is positive control (pig DNA extracted from venous blood). Lane 1 is DNA extracted from functional cell model with UH cell line, lane 2 is DNA extracted from functional cell model with BH cell line, lane 3 is DNA extracted from functional cell model with PSI cell line, lane 4 is DNA extracted from functional cell model with CLAB cell line, lane 5 is DNA extracted from functional cell model with C2 cell line and lane 6 is DNA extracted from functional cell model with PTC cell line.*

Primers designed for chicken species (GG_MTdl) amplified a 114 bp fragment in DNA extracted from cell lines in chicken functional cell models without showing any amplification in other extracted DNA (Figure 7).

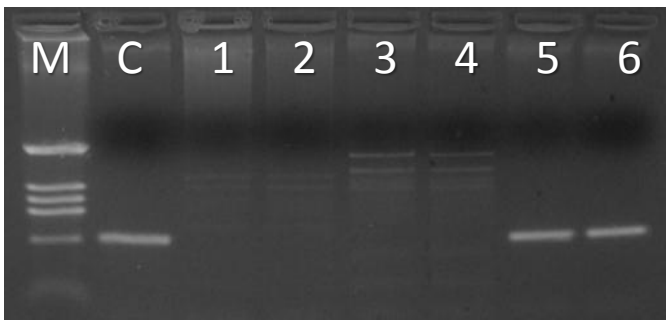

Figure 7. *Amplification of DNA extracted from functional cell models with GG_MTdl primer pair. Lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments, lane C is positive control (chicken DNA extracted from venous blood). Lane 1 is DNA extracted from functional cell model with UH cell line, lane 2 is DNA extracted from functional cell model with BH cell line, lane 3 is DNA extracted from functional cell model with PSI cell line, lane 4 is DNA extracted from functional cell model with CLAB cell line, lane 5 is DNA extracted from functional cell model with C2 cell line and lane 6 is DNA extracted from functional cell model with PTC cell line.*

In the first mixed functional cell model consisted of BH and PSI cell lines we can see that both primers designed for human (HS_MTdl) and pig (SS_MTdl) species were amplifying 169 bp and 645-648 bp fragments in DNA extracted from cell lines in mixed functional cell model but no amplification occurred with primers designed for chicken species (Figure 8).

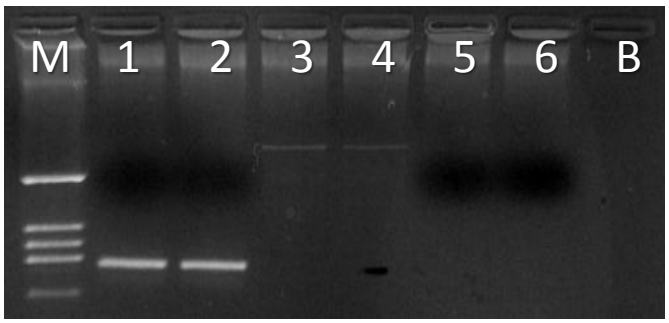

Figure 8. *Amplification of DNA extracted from deliberately mixed functional cell model containing BH and PSI cell lines with HS_MTdl primer pair, SS_MTdl primer pair and GG_MTdl primer pair. Lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments, lanes 1 and 2 are DNA extracted from BH/PSI mixed functional cell model amplified with HS_MTdl primer pair, lanes 3 and 4 are DNA extracted from BH/PSI mixed functional cell model amplified with SS_MTdl primer pair, lanes 5 and 6 are DNA extracted from BH/PSI mixed functional cell model amplified with GG_MTdl primer pair and lane B is blank.*

In the second mixed functional cell model consisted of UH and PTC cell lines primers designed for human (HS_MTdl) and chicken (GG_MTdl) species were amplifying 169 bp and 114 bp fragments in DNA extracted from cell lines in mixed functional cell model but no amplification was occurred with primers designed for pig species (Figure 9).

As expected, in the third mixed functional cell model consisted of CLAB and C2 cell lines, primers designed for human (HS_MTdl) did not amplify any product in DNA extracted from cell lines in mixed functional cell model but primers designed for pig (SS_MTdl) and chicken (GG_MTdl) species were amplifying 645-648 bp and 114 bp fragments in DNA extracted from cell lines in mixed functional cell model (Figure 10).

Conclusions

Our results indicated that the present developed PCR

assay can authenticate the cell line origin. Species specific primers designed in this study were targeting the mitochondrial *D-loop* region. The propensity to accept insertions and deletions makes the size of mitochondrial *D-loop* region highly variable in different and sometimes also closely related organisms (Sbisa et al., 1997).

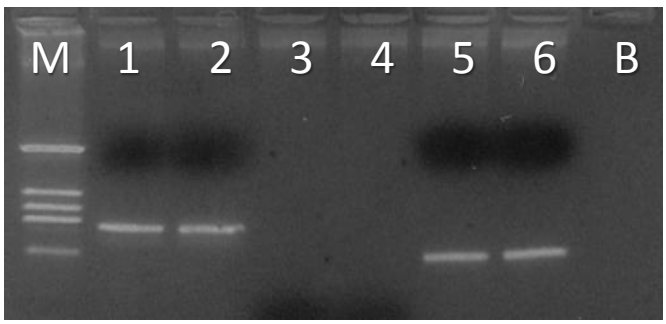

Figure 9. *Amplification of DNA extracted from deliberately mixed functional cell model containing UH and PTC cell lines with HS_MTdl primer pair, SS_MTdl primer pair and GG_MTdl primer pair. Lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments, lanes 1 and 2 are DNA extracted from UH/PTC mixed functional cell model amplified with HS_MTdl primer pair, lanes 3 and 4 are DNA extracted from UH/PTC mixed functional cell model amplified with SS_MTdl primer pair, lanes 5 and 6 are DNA extracted from UH/PTC mixed functional cell model amplified with GG_MTdl primer pair and lane B is blank.*

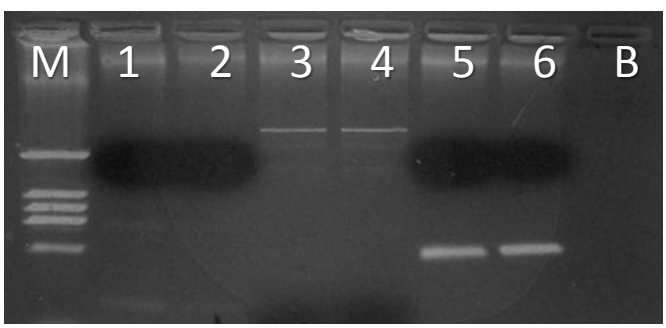

Figure 10. *Amplification of DNA extracted from deliberately mixed functional cell model containing CLAB and C2 cell lines with HS_MTdl primer pair, SS_MTdl primer pair and GG_MTdl primer pair. Lane M is DNA marker with 114, 176, 203, 245 and 500 bp DNA fragments, lanes 1 and 2 are DNA extracted from CLAB/C2 mixed functional cell model amplified with HS_MTdl primer pair, lanes 3 and 4 are DNA extracted from CLAB/C2 mixed functional cell model amplified with SS_MTdl primer pair, lanes 5 and 6 are DNA extracted from CLAB/C2 mixed functional cell model amplified with GG_MTdl primer pair and lane B is blank.*

The variability of mitochondrial *D-loop* region in different organisms provided us with high specificity of the designed primers, hence it follows that the mitochondrial *D-loop* region represents a powerful sequence for authentication of the cell line origin.

We believe that it is crucial to authenticate the origin of used cell lines and developed functional cell models while setting up an experiment involving cell lines, to be certain that there was no mislabeling of cell culture flasks or misplacement of cells from one to another cell line. With unequivocal PCR based authentication methods of the cell lines it is certain that our experiment handed us with relevant results typical for the cell line origin. It is also providing an incontestable proof that our experiment was carried out on the functional cell model from the same cell line origin as designed and stated in the experiment protocol.

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