Detection and Purification Methods for Environmental Inhibitors of Polymerase Chain Reaction in Forensic DNA analysis

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Abstract

Polymerase Chain Reaction inhibition is very common while dealing with the samples for Forensic DNA analysis. A lot of Forensic DNA samples are not useful for analysis as they have environmental inhibitors which may cause degradation of nucleic acid (DNA) which further leads to failure of amplification. The mode of action of these inhibitors is either attack on DNA or attack on polymerase enzyme. Environmental inhibitors include organic and inorganic inhibitors. PCR inhibitors may interfere at different steps of PCR analysis. Generally, inorganic inhibitors include Magnesium ions, Calcium ions, on the other hand organic inhibitors include bile salt, urea, phenol, ethanol, humic acid, tannic acid, melanin and protein. These different types of inhibitors can cause hydrolytic damage, oxidative attack, enzymatic reaction such as nucleases, bacterial attack can degrade the DNA and sun light may also effect in the same way. The purpose of this study is to identify the possible inhibitors for PCR present in environment which can pose problem during the amplification step of forensic DNA analysis, there mode of action and to find the best possible solutions for removing these inhibitors from forensic DNA sample.

Keywords: PCR Inhibitors, Detection Methods, Removal of Inhibitors

Introduction

The inhibitors are of many kinds which cause problem in PCR reaction during Forensic DNA analysis. These include heme from blood, humic acid from soil, dyes of cloths, nucleases from microorganisms, metal ions, gelatin and Ca2+ from bones etc [1–3]. These agents must be eliminated in order to get amplifiable human DNA. These inhibitors have various mechanisms to interfere the process of DNA typing.

Inhibition from Blood

Blood is crucial biological fluid often found at scene of crime as forensic evidence. It is good source of DNA used to solve crime puzzle. Often blood is mixed with soil after leaving suspects body and retains there for hours, sometimes for weeks, depending upon scenarios. Humic acid is a big problem for forensic analyst while dealing with soil mixed blood samples. Humic acid hinders with the detection system of RT-PCR and gives false negative (-ve) results by causing inhibition. Even in presence of high concentration of human genomic DNA, it hinders quantification process [4, 5]. Ct value of the sample increases in the presence of high concentration of HA leading to false detection of human genomic DNA [5]. This erroneous detection leads to increased or decreased input amount of template DNA in PCR which further leads to improper or false generation of DNA profile during genotyping.
Inhibition from Dyes of Cloths

Clothing and bedding are most important evidence exhibits from crime scene even if collected after several days. They have more importance than vaginal/anal swabs in sexual assault cases as seminal material could survive longer time on clothing. The only problem with clothing is presence of dye. Indigo dye is one of the most problematic inhibitor in crime scene evidence samples. During extraction of DNA from dark color fabrics or denim, colored extracted product gain which quench PCR dyes during RT-PCR analysis [6]. It will cause loss of efficiency of PCR dyes which further leads to false results (positive or negative with different chemistry).

Degradation from Nucleases

Nucleases are specific proteins which directly effects on nuclear DNA. These proteins come from microorganisms (mostly) and human being (rarely). These proteins work efficiently in moist condition. It can degrade DNA by chopping which lead to inefficient amplification and allele drop at genotyping stage.

Ca²⁺ Inhibition

Dealing with dead body identification is a continuous problem for forensic DNA analyst. In most of the cases, soft tissues from dead bodies are in the process of purification (early or last stage). The only chance left to obtain DNA for identification is bone. In most of the case scenarios, bones of dead body are treated for DNA extraction in which calcium is a major inorganic component [6]. It is positively charged ion which affects PCR process by Taq polymerase inhibition and also by competing with magnesium ions which leads to reduced efficiency of reaction.

Collagen Inhibition

Collagen is a protein present in body in different forms like rigid bones, compliant tendons and in between rigid and compliant like cartilage. Mostly it is observed when dealing with skeletal samples. Presence of collagen cause slightly reduction of Ct value during quantification of extracted DNA [6] because of binding template DNA which further affect process sensitivity of Taq Polymerase.

Melanin inhibition

Natural pigment found in living organisms. It is found in different parts like skin, hairs and eyes. It effectively absorbs light. In PCR, it binds sequence specifically with template DNA and limits the availability of DNA [6]. Its absorbance power can give false negative or reduced amount of DNA in RT-PCR analysis. It also effects Taq polymerase during primer extension.

Heamatin Inhibition

Heamatin is a derivative of hemoglobin formed after removal of protein part and oxidation of iron. It is a bluish-black compound with a red organic pigmentation. PCR inhibition directly depends upon its concentration. Increased amount of heamatin can fully inhibit PCR by attacking polymerase enzyme.

Environmental PCR Inhibitors

Many types of environmental PCR inhibitors are also present. The possible source, mode of action, targets and ingredients of these environmental inhibitors were studied by Mavziutov and Co. workers in 2003. Some of the inhibitors from environment decrease the sensitivity of the PCR method at different stages. These have also direct effect on polymerase enzyme, primers and also on DNA molecule [1]. There are many types of inhibitors for PCR present in environment and a forensic DNA analyst deals with these on almost daily basis. Micro nuclease, DNases and metallic inhibitors [1–3] are common when dealing with samples like putrefied dead bodies recovered from graves and from moist places.

Detection Methods for Degradation and Inhibitors

Taq-Man Technique

Humic acid (HA) is a big problem for a forensic analyst when dealing with such blood samples which are associated with soil. It is the major inhibitor for PCR process and gives false negative results even with a huge DNA quantity. For this purpose, Davorka and his Co. scientists in 2005 work on the efficiency of Taq Polymerase enzyme in the present of humic acid which introduced in forensic blood sample from soil and cause inhibition. ABI prism 7000 instrument was used with Taq-Man technique for
the quantification of blood sample. Samples was totally inhibited when 100 ng of HA was used partially inhibited when 10–75 ng of synthetic humic acid (HA) used [4].

qPCR Assays

Three different quantitation assays were introduced by Timken and his Co. workers in 2005 to observe degradation levels of forensic DNA samples with their detectable range. Targets from both nuclear and mitochondrial DNA was used for quantification purpose. Duplex nuclear-mitochondrial qPCR assay, ABI Quantifiler Human DNA Quantification Kit qPCR assay and slot blot hybridization. duplex qPCR assay gave better results, qPCR assay overestimate While in slot blot, quantification of sample was underestimated [7]. Quadruplex real-time qPCR” was developed by Hudlow and Co. scientist in 2008. It simultaneously quantifies the total human DNA, human male DNA, DNA degradation and PCR inhibitors for forensic DNA samples. It is helpful for choosing proper amplification technique when male to female ratio is very less (Y-STR analysis), or when degradation level is too higher to be amplified with Identifiler kit (Mini-STRs) [2].

Use of Thermus thermophiles

A wide range of thermostable polymerases (~10) were used for susceptibility of PCR inhibitors by Eilert and Foran in 2009. Out of all these polymerases, two enzymes; thermos aquaticus and thermus thermophilus have good resistance against PCR inhibitors while enzyme from Thermus flavus have low resistance against PCR inhibitors [8]. Barbisin and Co. workers in 2009 detect and quantify human DNA, human male DNA and possible PCR inhibitors in a single well with the help of cyclic threshold (CT-value). [9]. Humic acid (HA) inhibition effect was determined by Seo and Co. workers in 2012. They used a specific DNA concentration sample with the increased range of HA introduced. The Ct value is directly proportional to the concentration of HA for a certain limit. But when crossing 4.8ng of HA concentration, the Ct value was undetermined [5].

Microfluidics Digital PCR

Microfluidics digital PCR technology was firstly studied by Alaeddini in 2012. He studied different aspects of PCR inhibition through different environmental factors which includes humic compounds, soil, natural waters and recent sediments. He also studied different reports for proposed mechanism of inhibition, detection of inhibitors and assays for removing of these inhibitors [10]. Comparison was made between results obtained from conventional quantification of DNA and microfluidics digital PCR for the presence of inhibitors by Hoshino and Inagaki in 2012. The results showed that digital PCR technique provide accurate DNA quantification of sample with the humic acid concentration up to 9.3ng/μl while on other hand, other conventional methods did not give accurate results even with little amount of humic acid [11].

ALU Detection for Degradation Assessment

A new technique called “Alu detection” in DNA for the assessment of degraded DNA was 1st time introduced by Pineda and Co. workers in 2014. These are the short interspersed elements (SINE) consisting of 300bp present in human genome in very large amount (>1000 insertions). A short fragment of 80bp and a long fragment of 207bp are the targets for internal primer in quantification through InnoQuant (multiplex system). The difference between two quantitation values gives the estimation of degraded DNA as well as presence of inhibitors through IPC (internal PCR control) used. Alu targeting technique is better than the previous one used for estimation of contaminants and inhibitors because these are transposons genes and present in almost every human being in more or less abundance. So it is the easy target for quantification of human DNA with presence of inhibitors [12].

Quantifiler Trio Kit

A new Quantifiler Trio kit was used by Vernarecci in 2015 in addition to Globalfiler® Kit with 24 markers. This Quantifiler kit was applied on 181 environmentally degraded samples from real crime scenes. This technique is simplified, general and comprehensive [13].

Extraction Methods for Purification of Inhibitors

CTAB for Inhibitors Removal

Ye and Co. workers in 2004 introduce a novel technique for the removal of inhibitors and get a good and purified
yield of desired DNA. For this purpose, they used Cetyl Trimethyl Ammonium Bromide (CTAB) as a lysing buffer in extraction procedure of bones while at the end of lysis, Phenol-Chloroform Isoamylalcohol (PCI) was used for purification of DNA. DNA IQ system was used for the whole process [14]. Del Valle and Co. workers in 2004 different methods of extraction for degraded samples of bones affected by environment in different conditions method A (a modified method from FBI for bone extraction) gave better results as compared to other 2 methods. It gave better quality DNA but not a higher yield [15].

**Use of Silica Beads with Guanidinium Thiocyanate for inhibitors removal**

Rohland and Hofreiter in 2007. Used a method to maximize a purified DNA yield without further damaging. For this purpose, EDTA and proteinase K only were used for incubation and silica beads with high concentration of guanidinium thiocyanate was used for removal contaminants and inhibitors [16]. Ion exchange column was 1st time used by Am J Phys in 2008 for purification of extracted DNA from ancient bone samples. These bone samples were old about 500–3300 years and not preserved properly with PCR resistant chemicals with them considerably improve amplification success rate [17].

**Quick Spin Method**

Seo and Co. workers in 2010 studied different methods for extraction and purification of DNA from 60 years old bone samples. Methods involve partial demineralization, total demineralization, QIA quick spin columns and ion exchange columns. It was concluded that combination of total demineralization and quick spin column will give higher purified DNA yield with minimal inhibitors presence than partially demineralized method and ion exchange chromatography [18]. A novel technique was used for the 1st time in Tokyo by Kitayama in 2010 to extract DNA from degraded samples of bones and teeth without making powder. It involved conventional Phenol chloroform isoamyl alcohol (PCI method) gave full STR profile results [19].

**Inhibitors removal by SCODA**

Another method “Synchronous coefficient of drag alteration” (SCODA) was 1st time used by Schmedes and his Co. researchers in 2013 for the purpose of removing inhibitors as well as concentrates the sample to maximize the DNA yield. This method has the ability to effectively remove hematin, humic acid, melanin and tannic acid and gives no cyclic shift for IPC in DNA quantification. This is better approach to remove inhibitors from DNA samples with minimum steps involved [20].

**Nucleo Spin Method**

Nucleo Spin was introduced by Faber and Co. workers in 2013. It involves the kit name as “NucleoSpin DNA Clean-Up XS kit” which effectively remove eight (8) types of PCR inhibitors; bile salt, collagen, hematin, humic acid, indigo, melanin, tannic acid and urea. This kit contains silica membrane which bind all these inhibitors and only allows to pass purified DNA which is use full for STR amplification [21]. Seo and Co. workers in 2013 compare results of TaKaRa Ex Taq™ Hot Start Version and InhibitEX Tablet help to achieve better quality STR amplification results. Results showed higher resistance of TaKaRa Ex Taq™ Hot Start Version (Ex Taq HS) with BSA as compared with inhibitEX Tablet [22].

**Collagen Inhibition**

**Pressure Cycling Technique**

A novel technique “Pressure Cycling Technology” was introduced by Marshall and Co. workers in 2013. It was better technique than other previously used as it includes no chemical involvement with better STR results. The samples show degradation pattern in STR DNA profile due to hematin or humic acid will give better results with balance DNA profile after treatment with this technology [23].

**Microgel for low quantity DNA**

Geng and Methies in 2015 used the agarose microgel droplet approach for the purification of PCR inhibitors in single molecule of STR typing sample. DNA template purify through this porous gel by washing and significant dilution of targets. This technique showed increased
resistance to urea, tannic acid, and humic acid with many folds as compared to conventional extraction methods with tubes. Only those samples showed small inhibition which has humic acid concentration more than 200 ng/μL. This technique is useful for low quantity DNA with presence of inhibitors [24].

**Power Clean and Clean-Up Kit**

Four methods were used for the removal of PCR inhibitors by Hu and Co-workers in 2015. This study relates with research work done by Faber and Co [21]. The efficiency of all four methods was compared. Out of these four, two methods; PowerClean® DNA Clean-Up kit and DNA IQ™ were the most efficient in removal of eight common inhibitors; melanin, humic acid, collagen, bile salt, hematin, calcium ions, indigo and urea. The remaining two methods only remove some of these inhibitors. So the above said methods which can remove all inhibitors, should be recommend and use for environmentally compromised DNA samples [25].

**Types of inhibitors identified**

Forensic DNA analysis is the powerful tool for identification of individuals in different type of cases. It involves suicide, homicide, sexual assault, parentage, dead body identification, mass disaster and missing person identity. Forensic DNA analyst deals with these types of case on daily bases. Some of these cases involve the direct exposure of sample with environment such as blood from homicide and suicide case, skeletal remains from DBI and mass disaster scenes. These environmentally exposed samples face different problems like degradation and inhibition.

Degradation and inhibition are the major problems of DNA PCR which is being faced now a day by Forensic DNA analyst all over the world. 1st inhibitor (calcium ion) was reported in 1993 by Fisher [26]. Then nucleases were described as major factor of degradation by Mavziutov and Hudlow [1, 2]. Humic acid from soil samples was considered as the major source of inhibition at PCR level by Davorka [4], Hudlow [2], Fumes [19], Seo [20, 27], Hoshino [5], Schemeds [10], Faber [11, 24] and Marshall [20]. All the forensic DNA analysts deals with humic acid when processing the soil related samples in laboratory. Other inhibitors such as hematin, collagen, indigo, melanin, tannic acid, urea, metals and natural water also contribute in inhibition process of PCR [2, 10, 11, 19, 24, 27].

**Detection of inhibition in DNA samples**

Different techniques have been used in past for quantification of DNA. It includes Duplex nuclear mitochondrial qPCR, ABI quantifiler human, slot blot hybridization [7], quadruplex [2], light microscopy, SEM and elemental analysis [3] microfluidics digital PCR [5], pressure cycling technology [21] and Alu detection [22]. Some of these techniques detect inhibitors and degradation at a certain limit but some have the ability to detect at full level. DNA Analyst decides the protocol for amplification on the basis of inhibitor and degradation level. Sometimes DNA analyst observes false negative results due to severe inhibition or degradation which leads to wrong results in forensic DNA analysis. To avoid these practices, there should be a better technique for the detection of all types of inhibitors and severe degraded samples.

**Purification techniques**

After detection of inhibition and degradation, there should be a purification step for removal of inhibitors to maximize the purified yield of DNA for amplification step. Methods and techniques involved for purification of DNA are decalcification [26], CTAB with PCI [14], EDTA with Pk and Guanidinium thiocynate [16], ion exchange and size exclusion chromatography [3, 17], demineralization and quick spin [9], Nucleo spin [11], Powder clean kit and DNA-IQ kit [24]. Most of them can purify DNA at some extent while some of them are the best extraction kits which can effectively remove almost all the possible inhibitors and purify DNA sample at the level of STR amplification stage.

**Conclusion**

Duplex nuclear mitochondrial qPCR, ABI quantifiler human, slot blot hybridization [7], quadruplex [2], light microscopy, SEM and elemental analysis [3] were
used for detection of human DNA. All these above said techniques are effective in identification of degradation and inhibition for some extent but not at full level. Beside these techniques, microfluidics digital PCR [5], pressure cycling technology [21] and Alu detection [22] are used in now a day. These techniques effectively detect a better level of inhibition and degradation than other previous techniques. Decalcification [26], CTAB with PCI [14], EDTA with Pk and Guanidinium thiocynate [16], ion exchange and size exclusion chromatography [3, 17], demineralization and quick spin [9] are the conventional methods used for purification of DNA for a certain limit. These methods can purify DNA but side by side minimize the yield of DNA for amplification at the end level. So three effective techniques; Nucleo spin [11], Powder clean kit and DNA-IQ kit [24] are the best extraction techniques which can effectively remove almost all the possible inhibitors and purify DNA sample without lowering the yield for amplification at the level of STR amplification stage.

References


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