One step DNA extraction : buffer and method standardization study .

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Abstract :

Molecular techniques have been gaining importance in the field of diagnostics . The covid pandemic saw standardization of methods and kits for diagnostics in a faster phase. This paper deals with the standardization of method and buffers for one step DNA extraction. The process that requires both time and technical training in any molecular technique is DNA /RNA extraction. In this paper 2 buffers from the study of 13 different buffers with various combination and concentrations of chemicals were identified to be effective, quality and quantity of the DNA was seen from the A260/A280 ratio and the efficiency of the same was compared and confirmed with DNA gel electrophoresis and PCR techniques. These buffers in combination with techniques such as reverse pipetting, centrifugation , invert mixing were employed to check the efficiency of the protocol. Quantity of the buffers and samples were seen for the best results.

Introduction

The advent of the pandemic has shown leaps and bounds of interest and growth in the field of molecular biology and point of care testing. The first step to any point of care testing in molecular biology is DNA/RNA extraction (Wang et al. 2003). Often these steps are the most time consuming and require sophisticated equipments. This becomes the major limiting step in point of care testing. The search for quick extraction methods is progressing. This paper deals with the comparative analysis of various buffers and the use of varies chemicals and concentrations in the efficiency of the extraction. Many methods for DNA extraction from blood have been standardized ,however most of these methods employed hazardous chemicals or chemicals that needed storage (Proteinase k and Rnase).(Pachot et al. 2007)

DNA extraction is the first and basic step of any Molecular technique and was first successfully done in 1869 by Friedrich Miescher. However the density gradient centrifugation method that is modified and used today was standardized in the year 1958 by Stahl and Meselson (Dahm .R., 2005).The breaking down of the cell and nucleus to release out the genetic material and purify the same from other debrie such as protein is the main working principle of DNA extraction. DNA extraction techniques as known today have undergone voluminous testing and modifications. The popular techniquescurrently are chemical extraction, liquid –liquid extraction and solid –liquid extraction based. (Corkill, G., &Rapley, R) . The chemical extraction technique is broadly classified as organic and in-organic solvents based techniques.Common extraction techniques include Chromatography , Cs-Cl density gradient centrifugation , Phenol-chloroform &isoamyl alcohol , CTAB, Proteinase K, Spin column ,Magnetic bead based , paper DNA , anionic and guanidine thiocynate. Protienase k based methods provides high quality DNA , but storage of Proteinase K needs -20ºC (Tan, S. C., & Yiap, B. C. 2009).However most of these methods need sophisticated equipments , costly chemicals , involve many time consuming

steps and trained personal (Ali et al ., 2017). Hence this becomes the main limiting step in the use of molecular techniques in point of care testing.

Any DNA extraction method consists of 4 important steps 1. Cell lysis 2. Nuclear envelope lysis 3. Protein digestion and debris removal and 4. Precipitation of DNA.It can also be said as lysis , separation, precipitation, and purification (Hutami et al). A good DNA extraction method must 1. Maximize DNA recovery, 2. Remove inhibitors, 3. Remove /inhibit nucleases, 4. Maximize the quantity of DNA and finally 5. depending on the type of DNA needed for the specific method. The appropriate upstream preparation of DNA extraction is needed for molecular techniques. Liquid phase ad solid phase extractions are present for this (Boom et al).

Lysis steps may be carried out either mechanically, chemically, or enzymatically. Nuclear envelope lysis is required for the release of the DNA/RNA material. This step also involves the breaking down of proteins. Hence majorly in this step Proteinase K ,SDS or phenol and chloroform is used followed by centrifugation. The protein and te cell debris is further digested, separated and removed .Finally the DNA is precipitated using TE buffer or distilled water.(Gupta.N .,2019)

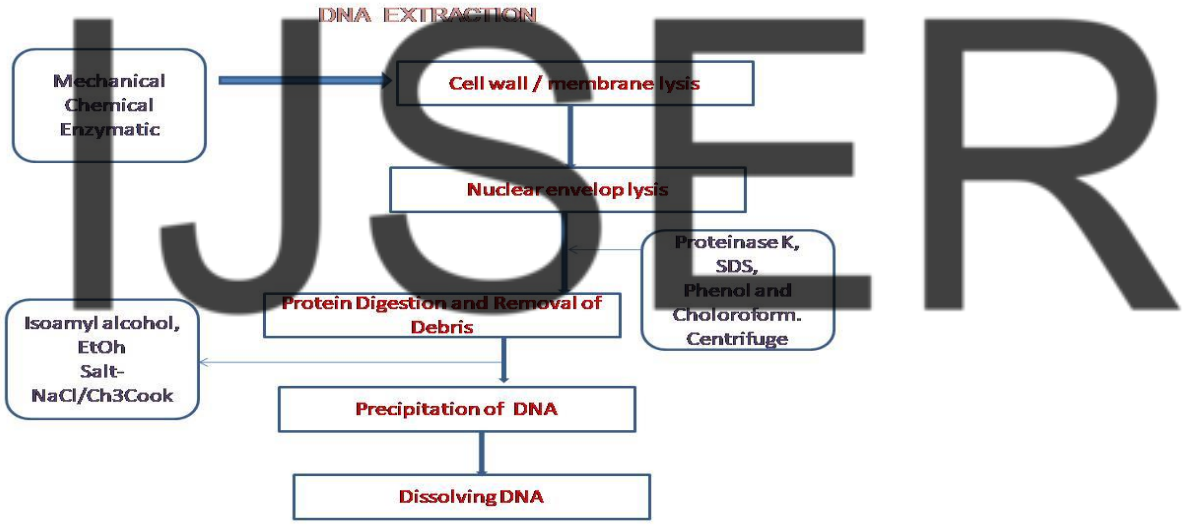

Fig 1 : DNA extraction steps

Components of the lysis buffer:

One major principle of extraction is the use of salts for creating a lysate and de –salting using ethanol for the release of nucleic acids in an aqueous solution. The purpose of the salts is to neutralize the charge on the sugar –phosphate backbone .Salts contain PO_4 and Na which are negatively and positively charged respectively and binding to the nucleic acids makes them less soluble by reducing hydrophobicity (Mohammadpour., 2018).

The lysis buffer based on the above principle contains salts that contribute to this ion donation, detergents to help rupture the membrane , proteinase k like chemicals that break down protein ,

ethanol to help the elution process, buffers that help preserve the DNA (Bienvenue et al.,2006). Some popularly used components of a buffer are:

Ethanol:

- In the presence of ethanol the Na and PO_4 interact with each other due to the lower dielectric constant of ethanol and hence helps with lowering hydrophobicity and hence the nucleic acid drops out of the solution(Green, M. R., & Sambrook, J. 2016).

Sodium chloride (NaCl)

- NaCl helps remove the protein bound to DNA and keeps protein in aqueous layer .It functions functions by neutralizing the negative charges on the DNA.
- Separate protein and carbohydrates from DNA (Gaikwad.,2002).

Ethylenediaminetetraacetic acid (EDTA)

- Chemically EDTA is aminopolycarboxylic acid$[CH_2N_2]_2$.
- EDTA is a chelating agent .The purpose in the buffer is the chelating of the metal ions present (Yagi et al., 1996).
- It works like the proteinase k and deactivates DNase and RNase activity.
- In the lysis activity, the lysis of the cell wall and nuclear membrane is aided by EDTA

Magnesium chloride ($MgCl_2$)

- Presence of $MgCl_2$ aids with the rupture of the cell membrane.
- Aids in the protection from DNase activity (Höss, M., & Pääbo, S. 1993)..

Xylose ($C_5H_{10}O_5$)

- Sugars such as Xylose mainly works in the breaking of the helical bonds .

Sucrose ($C_{12}H_{22}O$)

- Creates osmotic pressure outside the cell and breaks the cell.

Tetramethylethylenediamine (TEMED)

- Non –ionic detergent .
- Extracts proteins , organells by creations of permeability (Hansen,1981) .
- It helps in inactivation of lipid in enveloped viruses
- Usually in a lysis buffer it is added in a 5% alkaline solution .

TE buffer

- Helps with the preservation of DNA (Wang et al.,2007)

Sodium Dodecyl Sulfate (SDS)

- It is an anioinc detergent and aids with the linearization of protein (Nasiri et al.,2005).

Pottasium Chloride (KCl)

- KCl's activity in a buffer is to neutralize the charge present in the backbone of the DNA and aids with the better binding during PCR (Anker et al., 1997).

Pottasium acetate (CH3COOK)

- It is the Pottasium salt of acetic acid. It helps in the protein complex precipitation (Liu et al.,2000).

Polyvinylpyrrolidone **(**PVP)

- 1% PVP increases DNA yield.

Bmercapto ethanol

- Helps in protein reduction.

Pottasium hydrogen carbonate

- Helps with RBC breakdown.

Tris HCL

- Maitains the pH of the solution during the lysis process.

Triton X 100

- It is a non –ionic detergent and helps with the rupture of the cellular and nuclear membrane (Nasiri et al.,2005).

The touchstone for the evaluation of efficiency of DNA extraction is Gel electrophoresis a, DNA absorption ratio at 260/280 (wang et al) and comparison to the efficiency of the extraction with a *CE* approved kit.. Molecular techniques are used for a spectrum of samples from insects, bacteria, viruses etc. From the detection of the presence of the pathogen to study of the taxonomy, genetics , evolution molecular testing is used (schill et al). Each of the above samples / tests require different types of extraction techniques to get appropriate samples with maximum yield with high purity and stability but being cost effective (chambers et al). Each method used also varies in the DNA yield and hence may not be effective for quantitative techniques (Athanasio et al).Efficiency of the buffer increases with a lower number of chemicals and a stable pH.

This study deals with the preparation of buffers for single-step extraction of DNA from blood.Twelve (12) different buffers were made with different composition of the above mentioned salts and buffers and the evaluation of DNA purity was done with the help of nano drop and absorbance readings of 260/280 and 260 /230 were obtained. In the buffers that showed satisfactory purity the samples were evaluated with electrophoresis and DNA yield was also calculated. Methods to create variation were used in the buffers that showed promising results . Temperature , incubation time and lysis induction methods (no interference, invert mixing, reverse pipetting and centrifugation) were studied. The best results are reperesnted below.

Materials and Methods:

- Preparation of buffers: refer table below

Buffer 1: Tris HCL NaCl EDTA SDS 3%	Buffer 2: Tris HCL $MgCl_2$ Sucrose Triton X100	Buffer3: Tris HCL KCl $MgCl_2$ TEMED 1% Triton x 100
Buffer 4: Nacl $MgCl_2$ EDTA SDS KCl TEMED1% TE Buffer	Buffer 5: Tris HCL TE buffer KCl $MgCl_2$ EDTA SDS TEMED1% CH_3COOK PVP	Buffer 6: $MgCl_2$ EDTA SDS Triton X100 PVP CH_3COOK TE Buffer
Buffer 7: KCl $MgCl_2$ EDTA Sucrose SDS Triton x 100 PVP CH_3COOK TE Buffer	Buffer 8: TE Buffer Tris HCL 10 mm EDTA PVP SDS CH_3COOK Bmercapto ethanol Nacl	Buffer 9: TE Buffer Tris HCL 10 mm EDTA PVP SDS CH_3COOK Bmercapto ethanol Nacl CH_3COOK
Buffer 10: CH_3COOK Tris HCL EDTA SDS Ethanol	Buffer 11: CH_3COOK Tris HCL EDTA 1M $KHC0_3$ SDS Ethanol	Buffer 12: CH_3COOK Tris HCL EDTA 1M $KHC0_3$ SDS PVP Ethanol

Table 1 : Components of the buffers.

Various concentrations of the above chemicals were used to standardize the buffers. Invert mixing ,reverse pippeting and centrifugation steps were utilized to standardize the DNA extraction process and each step A 260/ 280 was taken to obtain purity. Ethanol wash and centrifugation was done on samples with RBC contamination / stored in -80^0C.Distilled water and TE buffer were used to precipitate the DNA. The results of the best are presented in the results section.

Absorbance at 260 nm, 280nm and 230nm.:
NanoDrop® ND-1000 was used to take the readings (Desjardins, P., & Conklin, D. 2010).

Quantification of DNA :

dsDNA concentration = 50 μg/mL × OD_{260} × dilution factor (Barbas et al., 2007)

Gel Electrophoresis:
Gel electrophoresis was carried out in a 1% gel . Only the best samples from the absorbance analysis were taken for electrophoresis.The gel pictures of the best results are represented in the results section. Running buffer used were TAE and TBE . Visualizion of DNA was done using EtBr through UV transilluminator and documented through gel documentation system (Lee et al., 2012).

PCR:
The main aim of this experiment is to standardize a buffer for DNA extraction in limited resource setting. Hence the DNA from the best 2 buffers were run in PCR to obtain the results and compared with that of a kit with CE approval for validation.

Ethical consent was obtained from

- Ethical approval for the project was obtained from HYCARE Wounds IEC
  Project no:027/HYC/IEC/2018 dated:13.12.2018
- Ethical approval for the project was obtained from HYCARE Super specialty hospital IEC
  Project no: 027 /HSSH-EC/2022 dated: 04.02.2022
- Samples used for this study were the excess samples (after regular testing) from the heametology department Regenix Super Specialty Laboratories The DNA so obtained from the process was only used to evaluate the results and discarded after analysis.

Results :
All experiments have been carried out in triplicate. The below results are the representatives of the same.
The purity of the DNA obtained is seen from the A260/280 ratio. A value between 1.7 - 2.0 is considered pure for ds DNA . A lower value indicated the presence of phenol, proteins and other contaminats as they are easily absorbed at 280nm . A higher value of the ratio shows the presence of RNase. In this experiment as proteinase K is not used to avoid the barrier of storage the need to rule out contamination becomes important. DNA Purity is calculated using the below formula and represented in the table.

DNA Purity (A_{260}/A_{280}) = (A_{260} reading – A_{320} reading) ÷ (A_{280} reading – A_{320} reading)
A260/230 is needed to rule out contaminants such as guanidine thiocyanate, guanidine HCL, triazole, phenol, etc. A ratio between 2.0 to 2.2 is considered pure for this ratio.
The below table contains the results of the nanodrop for the evaluation of the buffers: The results of the effective buffers are only represented in the table. buffers and methods which showed values of ratios below 1.5 or above 2.2 for ratios A260/A280 are not represented.

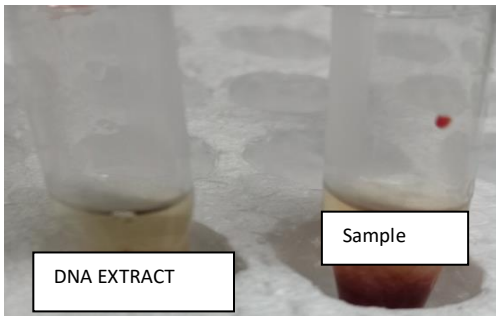

Fig 2 : representative image of DNA extraction from the buffer

S.no.	Buffer name	Method	A260/280	A2260/230	DNA Quantification ng/µl
1.	Buffer 3	A	1.68	2.2	4.1
		B	1.75	2.19	8.3
		C	1.52	2.17	5.9
2.	Buffer 7	A	1.72	2.15	4.3
		B	1.76	2.16	16.7
		C	1.78	2.12	25.3
3.	Buffer 9	A	1.72	2.17	18.2
		B	1.82	2.05	12.4
		C	1.85	2.11	32.8
4.	Buffer 10	A	1.91	2.09	36.5
		B	1.83	2.0	38.9
		C	1.86	2.14	31.5
5.	Buffer 11	A	1.85	2.02	36.9
		B	1.81	2.09	38.7
		C	1.88	2.12	35.1
6.	Buffer 12	A	1.86	2.1	40.1
		B	1.82	2.18	42.7
		C	1.85	2.09	35.2
7.	Contol	Kit Method	1.8	2.0	52.3

Table 2 : Nanodrop measurement and DNA Quantification

- A- Invert mixing
- B- normal –no interference

- C- Reverse pipette

*Centrifugation was required for methods B and C

*Ethanol wash and centrifugation improved the quality of the DNA. The results represented are after the ethanol wash.

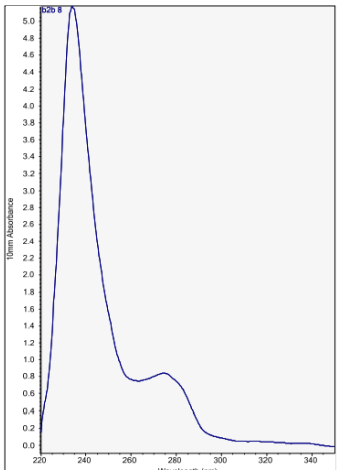

Fig3 : Nanodrop peak of pure DNA sample.

Heating step , centrifugation step were avoided for the use in remote resource setting. However, the buffers on storage needs to be heated at 65^0C for 5 minutes on storage before use. Various ratio of buffer to whole blood samples and buffers were tried out. The ratio of 200 µl buffer to 200 µl sample showed to be efficient , based on DNA quantity.The time for incubation was standardized to be 20 minutes using the best 5 buffers.

Buffers with PVP and CH_3COOK yielded good quality of DNA , while use of ethanol increased the quality and quantity of DNA . The reduction of salt concentrations and hence maintaining a stable pH of ~ 7.8 (5-9 – known to be ideal pH) showed better results .

Electrophoresis

The results of the gel electrophoresis shows that, despite good ratios of A260/280 the DNA quantity was lesser and hence very faint bands were seen (gel picture 1). However the buffers with effective values showed good quantity of DNA and a single band showing effective extraction procedure (gel picture 2). The results were comparable with that of the extraction of DNA done by kit method . A 100- 1000 bp ladder was used to compare the size of the DNA.

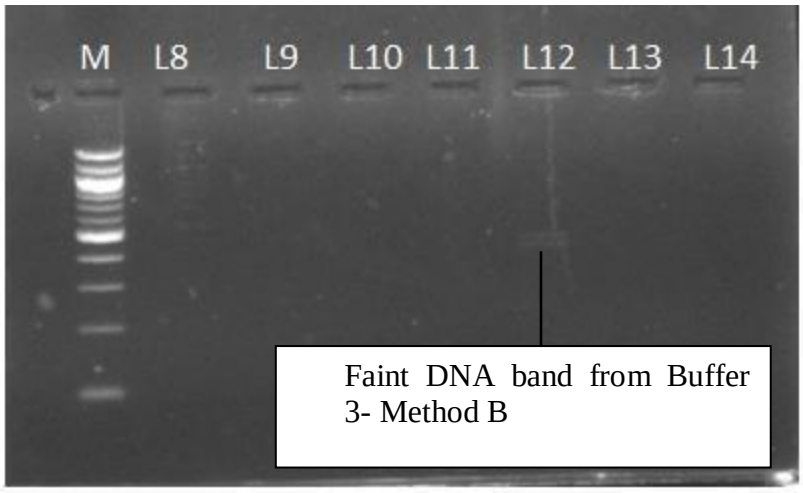

Fig 4: electrophoresis - Gel 1

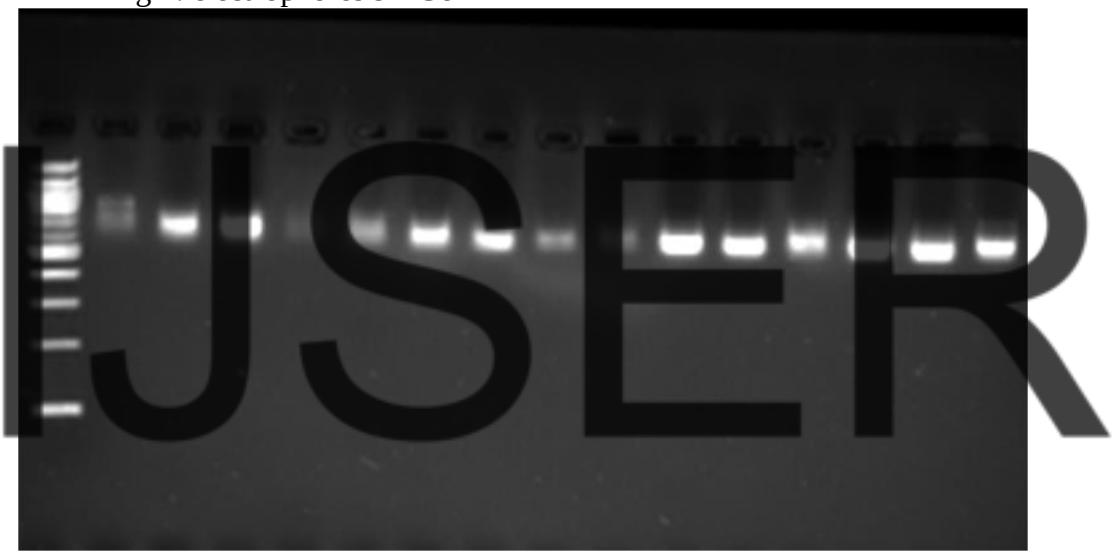

Fig 5 : Gel 2: 3 sample from the effective 5 buffers.

Lane 2 B7C	Lane 5 B9C	Lane 8 B10A	Lane 10 B11 A	Lane 13 B12 B
Lane 3 B7B	Lane 6 B9B	Lane 9 B 10 C	Lane 11 B11 C	Lane14 control kit method
Lane 4 B9 A	Lane 7 B10 B	Lane 10 B11 B	Lane 12 B12 A	Lane 15 B12 C

PCR:

PCR was conducted as a pilot study to see the effectiveness of the buffer in the use of PCR. Two sample were run with the buffer and DNA extract from an CE approved kit. One sample a known positive for a gene and other known negative. The results showed good Internal control values for both the samples using the buffer , attributing to the fact positive DNA

extraction was carried out. The positive sample showed positive owing to the buffer's sensitivity and negative sample showed NTC supporting to the buffer's specificity.

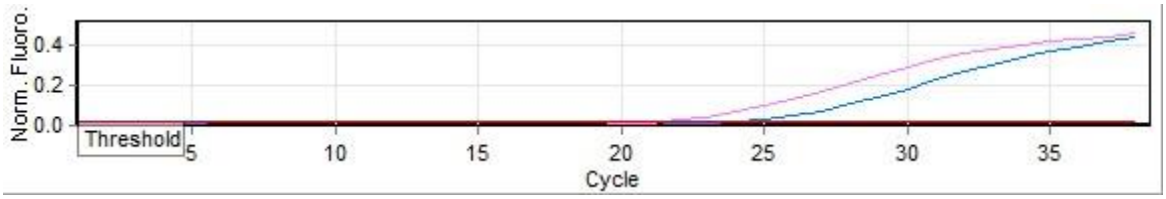

Fig 6 : FAM channel showing positives for HLA Positive samples . Pink –CE kit. Blue – Buffer.

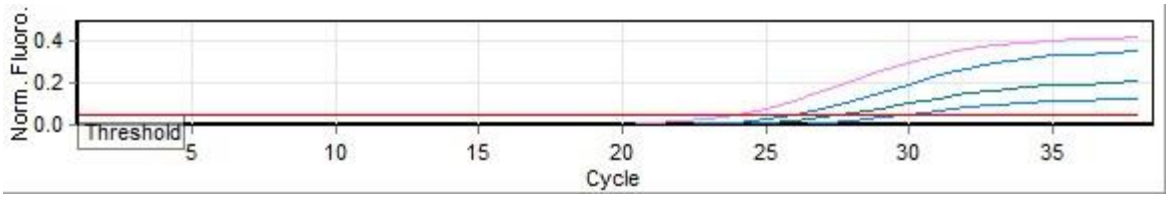

Fig 7 :HEX channels showing Internal control where the negative sample are also seen

Discussion:

An effective method of DNA extraction is one where DNA of good quantity and quality is extracted for use in molecular biology. The challenge in using this highly sensitive technique in the field is the time , sophisticated equipments and storage limitations. This work is an humble attempt to conquer that barrier in field settings. Time and trained personel's need is easily eliminated in this process. DNA needed for analysis is between 0.1-1ng of plasmid DNA .If pure DNA of this quantity can be obtained then one major challenge to use molecular techniques can be overcome.

Conclusion

This study is a pilot study to evaluate an efficient buffer and method for DNA extraction in limited resource settings. This study is the first step to making molecular testing available for screening in limited resorce setting and reducing the turn around time. The above buffer was successfully evaluated for use,

Conflict of Interest

The Authors report no conflict of interest in this paper .

Funding Statement

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