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Method

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Simplification of Molecular Methods

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Abstract

We have simplified and improved few techniques in furthering applications of molecular methods and products. This has been largely achieved by making them simple, ambient stable, rapid and cost effective.

In this comprehensive article, beside simplification of few methods, we are reporting on devices like a "home-made cheap ultra-rapid thermal cycler" and a "homemade cheap and efficient Portable Transformation Device" for high efficiency transformation of one millilitre of fresh as well as stored cultures. Included in this paper are methods of extended storage of DNA at Room temperature in a ready-to-use form and for repeated utilization; development of ambient stable, rapid and cheapest in the world DNA Diagnostic kit for identification of micro-organisms; plant sex determination at nursery stage under Field as well as laboratory conditions. The simple kit can be used even by semiskilled personnel. Also reported here are sensitive and rapid detections from clinical samples; cheap and easy methods of DNA synthesis; easy, cheap, rapid and large scale DNA isolations. These methods can have wide applications in detection of infection and surveillance during outbreaks, pandemics, and as a routine exercise in the Border areas impending threat of Biological Warfare.

Keywords: DNA immortalization; DNA Diagnostics as a Field kit; Efficient DNA synthesis; Homemade ultra-rapid thermal cycler; Homemade Portable Transformation Device; Sensitive and rapid detections from clinical samples.

Introduction

The wide applicability of molecular methods in entire life-sciences due to accuracy, uniform procedure, amenability to *In-silico* technology and automation, make molecular methods the choice technology.

We have attempted to simplify few molecular methods and develop few simple devices for isolation, storage, sensitive and rapid detection, surveillance, manipulation, and amplification and DNA synthesis. The simplified and improved molecular methods cover from immortalization of DNA to development of a cheap and ambient stable DNA diagnostic Field kit for use by semiskilled personnel and also development of devices like homemade ultra-rapid PCR and a homemade portable Transformation device, both costing a dime.

This comprehensive paper covers improvement and extension of molecular methods for further wide applications. It is expected to have special significance in detection during the on-going COVID-19 pandemic (by ultra-rapid PCR and stable and rapid Northern blot). Several of the methods reported, can be utilized directly for accurate, rapid and cost effective detection as well as in large scale surveillance program. It can be used for sex determination of plants like banana, papaya, sea buckthorn etc. as early as at one or two leaf (nursery) stage on Field thereby dispensing waiting till the fruiting stage.

The methods and devices include

Immortalization of DNA in a ready to use form for infinite shelf life under ambient conditions.

Ambient stable and cheap Surveillance Kit for Field applications.

Homemade Thermal Cycler for Ultra-Rapid detection of pathogen.

Cheap, rapid and efficient Transformation device for transformation of 1 ml of fresh or stored cultures.

Efficient, easy and inexpensive DNA synthesis.

Sensitive detection of microorganism from clinical samples.

Rapid detection of infection.

Easy, cheap and rapid isolation of DNA.

"Immortalization of DNA in a ready to use form" for infinite shelf life under ambient conditions

DNA gets degraded either by formation of nicks due to repeated freezing and thawing or by the different exonucleases and endonucleases like the deoxyribonuclease, Restriction endonuclease, S-1 nuclease etc. which are present [1-7] as contaminant in several other commercial enzyme preparations and in contaminating bacteria. Examples of DNA damage by bacterial contamination can be found in the report that enzyme nucleases make up 80% of the cell wall in *Flavobacterium sp.* like *Staphylococcus aureus* [2]. It is also reported 4 that the total cellular DNAase activity in the cell wash, cell-wall compartmentalized, intracellular fractions and culture supernatant contained are 27.8, 27.8, 13.1% and 31.3% respectively in *Clostridium pasteurianum* NRRL-B598.

All the above enzymes however are known to be dependent on cofactors like Mg+2, Mn+2 or Ca+2 ions for their activity. These cofactors can be chelated by appropriate concentration of sodium citrate [1,5,6,7] making them unavailable for degradation of DNA by contaminating enzymes. We have worked out a concentration of trisodium citrate at which DNA under repeated use, can be preserved infinitely under different room temperatures [1,5,6,7] and even at 37°C or more [1, 5]. We have preserved DNA at room temperature for over three years in a parafilm wrapped Eppendorf tube under air conditioning (8 h a day, for 5 days a week) and have used it occasionally for research. The DNA after over three years was found to have been dried up due to slow evaporation from the tube. However, upon resuspension, the DNA was found intact.

This simple buffer for dissolving and storing DNA at Room temperature consist of

Sodium chloride (NaCl)-0.02 M

Trisodium citrate ($Na_3C_6H_5O_7$)-0.06 M

PH= 7.0–7.2

We have later verified the method by challenging DNA with DNAase-I (0.4 units of DNAase-I for every 30 µl of DNA and RNA pellet dissolved in the above solution) [5,6]. It was observed that the DNA was intact (Figure 1). Hence we concluded that all the nucleases were inhibited.

We also have tried various other concentrations of trisodium citrate ranging from 0.02 M to 0.05 M and observed that protection (enzyme inhibition) was not absolute.

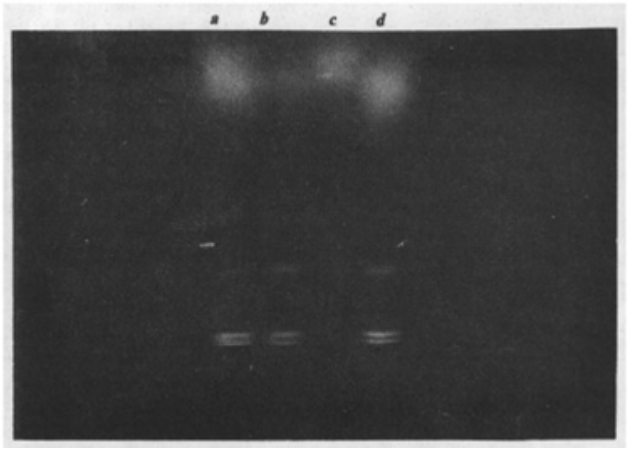

Figure 1: Inhibition of DNase activity. DNA was challenged with 0.4 units (excess quantity) of DNase-1(M/S SigmaUSA)at room temperature. DNA and RNA pellets were dissolved in the new buffer (lanes 'a' and 'b') and also in the conventional Tris-EDTA(TE) buffer in (lanes c andd). DNA is found protected in the new buffer (lane b) after challenging with DNase-1 but got degraded in TE (lane 'c') buffer. Lane 'a'and'd' are the two buffer controls.

The stored DNA can be used as template for DNA hybrization and other purposes. However, a few applications may require further dilution and even resuspension in water.

Ambient stable and cheap dna detection/ Surveillance kit for field applications

Our work on immortalization [1] of DNA has been utilized for development of DNA Diagnostic or Surveillance kit for Field applications [8]. The ambient stable kit contains aqueous stable DNA [1,5,8] both as probe and as template (from test sample), the hybridization as well as washing solutions as dry powder and detection under UV torch. Under the above conditions, the kit is expected to have an infinite shelf life and the cheapest diagnostic kit in the world as it contains exclusively or largely the inorganic components. Our earlier studies have revealed encouraging results.

The method also dispenses the need for sterilization 8 and pH adjustment [8] of any of the required solutions under 'Field conditions'. Sterilization is dispensed due to inhibition of DNA degrading enzymes (as in above section). Adjustment of pH for the final volume of the hybridization and washing solutions are made at source by addition of appropriate quantity of citric acid ($C_6H_8O_7$) powder to the mixture powder or its solutions which contain sodium chloride (NaCl), trisodium citrate ($Na_3C_6H_5O_7$) and sodium dodecyl sulphate (SDS), all as dry powder. While adjusting the pH of the prospective solutions, we are to keep in mind the final molarities of Na+ in the end volumes. The powder stock is supplemented with 0.3% or more of sodium dodecyl sulphate (SDS or lauryl sulphate) for use in hybridization and washings which additionally destroy any microbial or enzyme contamination. Dissolved in the hybridization solution during use (if required) either fat-free milk powder or fragmented denatured salmon sperm DNA or Denhardt's reagent as mixed powder can be added to the hybridization solution to block the available DNA binding sites on the membrane. However this step can be dispensed by methods like use of M/S Whatman 541 filter paper (Sl. No. vii) which has several other advantages discussed later.

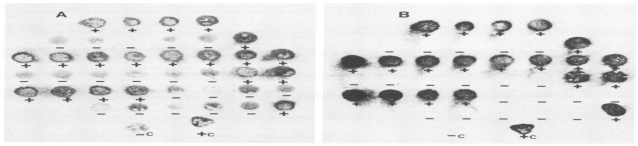

Figure 2: Whatman filter paper containing EHEC and other E. coli colonies hybridized with the EHEC DNA probe. (A) Filter not pre-treated with lysozyme-sucrose or proteinase K; (B) filter pre-treated with lysozyme-sucrose followed by proteinase K. Source: Gicquelais, et al (1990). Practical and Economical Method for Using Biotinylated DNA Probes with Bacterial Colony Blots to identify diarrhoea-causing E. coli. Journal of Clinical Microbiology, Nov. 1990, p. 2485-2490. Permission for reuse of data received.

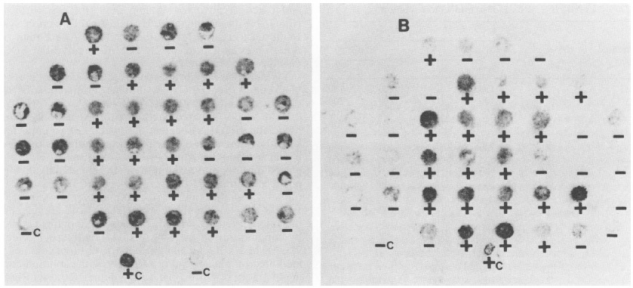

Figure 3: Whatman filter paper containing EIEC and other E. Coli colonies hybridized with EIEC DNA probe. (A)Filter not pre-treated with lysozyme-sucrose or proteinase K; (B) filter pre-treated with lysozyme -sucrose followed by proteinase K. Source: Gicquelais, et al (1990). Journal of Clinical Microbiology, Vol. 28, No. 11. 2485-2490. Permission for reuse of data received.

The probe used can be labelled or tagged with fluorescent dye like SyBr Green, DAPI (4′,6-diamidino-2-phenylindole), or 7-AAD (7-aminoactinomycin D), Hoechst 33258 (33342, 34580), auto-fluorescent quantum dots, Taqman oligo probes etc. for instant viewing of result under UV lamp/ torch.

Solid-state-supported approach is widely used in DNA chips, microarrays, and DNA sensor technology [9,10] as this heterogeneous hybridization format with a probe or the analyte attached to the solid support which facilitates the easy separation of hybridized and unhybridized oligonucleotide probes [8], simply by washing. This also enables screening large number of samples easily at the same time and cost. Thus it is suitable for all surveillance studies. We recommend the

use of Whatman 541 or the charge modified Nylon membrane as the solid matrix for this study.

For early (Nursery stage) sex determination in plants like banana, papaya, sea buckthorn etc., 5% Teepol detergent solution can be added to the crushed leaf cell for lysis before spotting directly on the membrane. The immortal DNA probe is chosen from the sex linked Sequence-Characterized Amplified Regions (SCARs) in plants. This approach in the above plants, dispenses prolong waiting till the fruiting stage.

Homemade thermal cycler for ultra-rapid detection of infection.

This simple homemade cheap device as well as the protocol enables rapid (5 minutes) amplification of a specific sequence of DNA by polymerase chain reaction (PCR). This Thermal cycler can be self-assembled at home or in the laboratory.

The Thermal cycler to be used can be programmed or operated manually as the run time is only 5 minutes. It consists of a powerful Tungsten lamp (500 Watts to 1000 Watts depending upon the size of the container box) for instant heating inside a small preferably transparent thermoplastic or metallic box. This box is also having a hole at the bottom. The hole at the bottom may remain open permanently or have a lid or flap opening inside under vacuum suction from top of the device (Figure 4). Forced evacuation of hot air from the top by a vacuum pump results in instant cooling of the reaction. Instant heating or cooling of the reaction is also possible because of the thin-walled glass capillary tube or thin 0.2 ml PCR tubes used which have large exposure area but contain a small reaction volume (5 µl to 15 µl). This enables rapid and uniform heat-cold alterations. The vacuum pump is connected at the top of the device for rapid evacuation of the hot air drawn from the hole at bottom during suction.

The device comprise of a lamp, a vacuum pump for suction from top and a bottom holed box without or with a lid opening inside for evacuation of hot air during suction. The device can be programmed for alternate 'On' and 'Off' of the lamp and pump during the heating and cooling phases. The bottom hole can even remain permanently open dispensing the lid as the hot air goes up only. These two operations can be programmed or controlled manually as the run time is only 5 minutes.

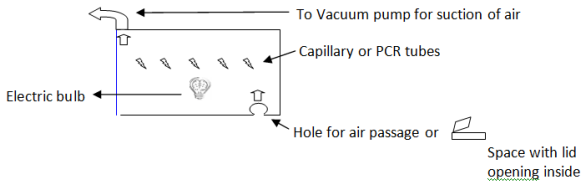

Figure 4: Homemade ultra-rapid thermal cycler

The mixture of PCR reaction consisting of template DNA, primer sequences, enzyme Taq DNA polymerase, DNTPs and Sybr Green [10] dye get sucked automatically into a thin-walled glass capillary tube or pipetted into the 0.2 ml PCR tube. The tube is closed (or heat-sealed at either ends in case of capillary tube) with the content for PCR and put in the above assembled device for DNA amplification.

This Rapid PCR protocol uses the following schedule for brief heat denaturation of 1- 2 min as the first step, give no time (0 Sec) during the next 2 step (strand separation and primer annealing) during later cycles due to the use of instant heating and cooling dispensing ramping time in an extended exposure area with small reaction volume. A brief period of 6 sec is given in each cycle for DNA synthesis by chain extension. Final extension of 30 sec is given for completion of all the synthesis work.

Typically, it operates as

Rapid method:

1 x Initial denaturation -1-2 min

Denaturation -0 sec

30 x Primer annealing -0 sec

Extension of chain -6 sec

1 x Final extension -30 sec

This is in contrast to the Standard PCR detection which takes from 3 h to 5-1/2 h for completion of 30 cycles and then the result is viewed later by electrophoresis, staining (and destaining) and screening under UV lamp, all requiring additional time.

Confirmation of successful DNA amplification can be made instantaneously by observing the green coloration of PCR products through naked eyes due to the intercalation of Sybr Green dye in the freshly synthesized DNA duplex. The change of colour can be observed directly after 5 minutes during the process of cycling if the box is transparent or by removing the tube during the run. Completion of 30 cycles however takes a total of 8 min and 30 sec.

This rapid, simple and inexpensive method dispenses the need [3] of compressor or peltier based Thermal cycler or microfluidic reactor (chip) capable of flow-through PCR etc. which are expensive, time consuming or are vulnerable to metal fatigue.

This cheap and simple device and the rapid protocol is made possible through the:

Keeping the whole or gap in bottom open or fitted with a lid opening inward under vacuum suction from top for instant cooling. This dispenses the associated technology for opening and closing.

Instant heating by a Tungsten bulb and instant cooling by vacuum pump nullifies any ramping time.

Dispensation of time consuming and expensive compressor or peltier based heating and cooling devices.

Instant viewing of result dispensing the need for electrophoresis for checking result.

Our simplified version costing a dime can be made at home or any laboratory or unit across the world and operated manually or be programmed.

Cheap, rapid and portable device for Transformation of 1 ml of fresh or stored culture.

An inexpensive (Rs. 1300/ or $ 17.12), rapid (1 minute) and portable transformation device has been designed and fabricated (Figure 4) by us in the laboratory [11]. This device enables transformation of even long DNA segments and dispenses the requirement of large quantity of fresh culture at log phase-3 and making of cells competent 3 by a time consuming processes of chemical treatment with extremely precise heat-cold shock of thermal

regimes [3]. It also dispenses the need for expensive electroporator devices. We have used this device for transforming a small volume (1 ml) of old culture stocks (having taken out directly from refrigerator) and transformed in 1 min time with high efficiency [11].

Several methods of transformation have been developed for research and commercial purposes. A contemporary method is magnet-assisted transformation which is almost exclusively used for the transformation of animal cell cultures. This is based on directing any ferromagnetic or paramagnetic DNA binding particle that can be used for this purpose. We have used the super-paramagnetic iron oxide nanoparticles (SPIONs) tagged with the desired genetic material using a static magnetic field. This versatile method which can transform various types of cells, involves a dynamic magnetic field that induces acceleration of nucleotides conjugated SPIONs and consequently cause them to penetrate the cells with robust barriers like the cell wall, peptidoglycan layer etc.

Our method contains alternating magnetic field placed as radials to guide and accelerate the SPION particles (10 nm-100 nm size) to attain necessary momentum for penetrating and thus transforming the cells with a desired gene.

The magnetic field surrounding the gap in which an Eppendorf tube is placed in the device will result in the acceleration of SPIONs suspended in above mentioned (at Sl. No.1) immortalization or any DNA buffer in the tube. In this particular system, the electromagnets get activated individually and sequentially in a consecutive radial fashion, causing the SPION-nucleotide complex to move along with the rapidly changing direction of magnetic field. By manipulating the time interval, the particles can either be accelerated or decelerated. The host cells to be transformed along with the SPION-nucleotide complex are put together in the immortalization or any buffer and can be placed in a 1.5 ml Eppendorf tube. By switching ON the device, the SPION-nucleotide complex will penetrate the cells. In practice, the particles, DNA and cells to be transformed are put together in the immortalization buffer from beginning of transformation.

Our SPIONs were coated with a cationic polymer, polyethylenimine (thus pSPIONs) for better binding with the DNA. Once suspended and magnetic field is switched ON and movement of pSPIONs will shift from Brownian motion to the field direction. Hence by controlling the magnetic field pattern and strength along with the size of pSPION particles, the motion and momentum of particles can be manipulated and empirically optimized for transforming a wide variety of different types and strains of cells.

Transformation of the host cells was continued for 1 and 2 min. The cells then were plated on selective media and growth monitored.

We have tested this device for efficient gene delivery [11] and transformed E. coli DH5α cells with a large 6.5 Kb DNA plasmid (amp marker) in as little as 1.0 ml of 4°C stored cell culture in one minute [11].

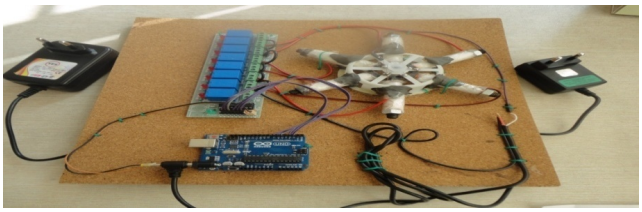

Figure 5: Portable Transformation device costing INR 1300 (USD 17.12)

The specification of the device developed is as under.

Micro controller: Arduino Uno R3e

Relay Board: Eight channel (8 ch) 12 V based relay board module: switch frequency 0.1 sec.

Electromagnets (0.5 Amps): 6 Solenoids; 0.5 in (diameter) and 2.5 in (length) bolts wound with 0.25 mm enamelled copper wire.

Power source: Relay - 19.5 V; 5 Amp

Control module: 5 V; 0.5 Amp

Electrodes: 12 V; 1 Amp

Control module switch frequency: 0.1 sec

Efficient and inexpensive DNA synthesis

We are reporting a method for synthesis of DNA which is simple, efficient, controllable and cost effective [12].

Among all the methods of DNA synthesis3 the Multi-prime DNA is by far the most widely used method which uses the random hexanucleotide (6N) primers along with the dNTPs and the enzyme Pol –I (KF). However, this method has the following problem, which can be resolved as under.

Criticality involved [3] in random primer extensions for synthesis of DNA chain or labeling is due to the thermodynamics of oligo-annealing and synthesis by polymerase enzyme which had to initiate simultaneously.

Annealing of the 6N oligonucleotides occur in ice (0°C) and DNA synthesis by enzyme large Klenow fragment (Pol –I KF) needs 37°C temperature.

The Standard Protocol for random primer extension follows:

Denaturing DNA by heating at 100°C for approximately 10 min or more, rapid cooling in ice, addition of random hexanucleotide primer while keeping the denatured DNA template in ice and then prompt shifting to 37°C for extension of chain for 30 min or more.

The proposed method follows:

Sequential harnessing of the catalytic and synthetic activity of the enzyme Pol–I (KF). We have first used [12] the 3'-5' exonuclease activity of Pol–I (KF) on both strands of DNA (heat denaturation is not required) simultaneously at 37°C in standard reaction buffer but without the NTPs. This incubation for exonuclease activity at 37°C was for few seconds or more depending upon the length of DNA and reaction conditions. The process generates self-primers of different lengths on both the strands of DNA, thus dispenses the need for random hexanucleotide primers and denaturation of DNA [13] has performed extensive study on speed of exonuclease activity of the enzyme. Controlling of exonuclease activity can be done by adjusting time, temperature or concentration of reactants.

Finally harnessing the 5'-3' polymerase activity of Pol–I (KF) is done simply by the addition of 2 nm each of all the 4dNTPs to the reaction at the same temperature (37°C) for extension of generated primer. For probe generation, 16 pm of one-of the labeled dNTP3 need to be added in addition to 2 nm each of all the 4 unlabeled dNTPs.

This is possible because the Pol 1(KF) has only the 3'-5' exonuclease and the 5'-3' polymerase activities. In the absence of the building blocks or the dNTPs, only the 3'-5' exonuclease activity gets initiated and the polymerase activity remains suspended. However, as soon as the dNTPs are added, the polymerase activity predominates.

Therefore by sequential harnessing of the 3'-5'exonuclease and 5'-3' polymerase activities of Pol -1(KF), we can first generate self-primers of varying or desired length on both the strands of DNA simultaneously which is followed by synthesis of DNA in the same reaction by addition of dNTPs.

Advantages of the method

Synthesis or labeling both strands simultaneously.

DNA labeling at high as well as controlled intensity.

Better signal to noise ratio.

Primers of different length can be self-generated at no cost.

Dispenses the need of 4096 combinations of random hexanucleotide primers.

Dispenses the need of heat denaturation of DNA

Complete operation at 37°C.

Dispensing the criticality of oligo-annealing in ice and DNA synthesis at 37°C.

Generates long as well as short DNA fragments.

Simple, efficient, cost-effective and fail-proof method.

With respect to the speed of exonuclease and polymerase activities, 'Rapid quenching and stopped-flow bulk techniques' have determined that during primer extension synthesis, the first nucleotide is incorporated by E. coli Pol- I (KF) with a rate ranging from 40–87 nt/sec while subsequent nucleotides are incorporated at a slower rate of 13–15 nt/sec14. The bulk processivity however, is twice as fast as compared to that found for single molecule primer extension synthesis (7 nt/sec). Primer extension synthesis is also reported to be faster than strand-displacement synthesis and this idea is supported by a bulk rapid quenching study which found that the strand displacement synthesis rate of Pol-I (KF) was even slower at 1.2 nt/sec [14].

The exonuclease activity of the wild-type T4 DNA polymerase has been reported to be 65.1 nt/sec in single-stranded DNA while 0.16 nt/s in a 16 to 24 mer DNA double strand [14]. Therefore, depending upon the length of the probe (which affect the labelling intensity thus sensitivity of detection) or primer required, we can decide the duration of exonuclease activity. The speed of enzyme activity can also be regulated additionally by the above mentioned methods.

This method is superior to DNA synthesis by chain extension using random primers as it dispenses the need for random primer, denaturation of DNA, different temperatures and also the criticality involved in the thermodynamics of oligo-annealing at 0°C and initiation of chain extension [12] at 37°C. It can generate longer probe and higher labelling intensity for more sensitive detections. Besides, this method is cost-effective as it dispenses the need of 4096 different combinations of (random) primers.

We have found the method is efficient, fail proof, controllable and cost effective [12]. The method works even when the DNA has been cut out from low melting point (LMP) agarose and used for labelling directly, dispensing elution and re-precipitation of the excised DNA.

It is noteworthy that the length of primer is related to the labelling intensity, signal-to-noise ratio, sensitivity and specificity of the DNA probe, therefore we can exercise options for facilitating diagnosis.

Sensitive detection of microorganism from simulated clinical samples

We have detected [15,16] as low as 8 bacteria (Figure 6) from 1 g of stool sample by the following method.

For this study, we have taken 1 g of stool sample which was mixed thoroughly with 3 ml of sterile normal saline (SNS). Simulation of clinical sample has been accomplished by thoroughly mixing the above stool sample in SNS with 20 µl of overnight culture of E coli with a 2.8 kb pDAS-101 plasmid (having157 bp of ST-1a E. coli enterotoxin gene insert and an 'amp' marker).

The mixture was centrifuged at 2500 g for 10 min and 20 µl of supernatant was collected and this was further diluted with 10 ml of SNS. The diluted sample was spotted on nitrocellulose membrane at decreasing concentrations from 390 µl to 01 µl as discrete spots by a Dot Blot apparatus under vacuum suction and hybridized with 32P ST-1a DNA probe [15,16].

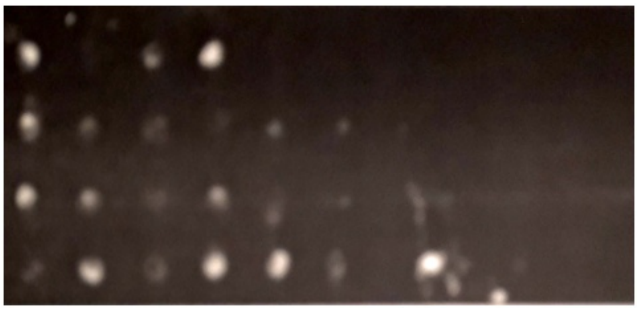

Figure 6: Autoradiograph showing sensitive detection ETEC directly from stool sample. Hybridization was seen in all points above 100 µl application spot, indicating 8 or more ETEC. Source: Lahiri and Karothia (1993); Sensitivity of detection of enterotoxigenic Escherichia coli from stool by DNA probe. Acta. Microbiologica Hungerica. 40(1).59-63. (Permission not required).

Viable cell count of bacteria on ampicillin plates revealed that the minimal volume of highly diluted sample detected by dot-blot [15,16] contain 8 bacteria/g of stool sample (Figure 6).

Rapid detection of infection

The method performs DNA hybridization on M/S Whatman No. 541 filter paper [17-24]. The filter paper chosen, is made of cellulose acetate [17,20] which is non-fragile, soft, easy to handle [17,18,20,22] cheap, soaks and wet easily, dispenses the need for vacuum baking or UV cross linking, any pre-treatment [17,18] (except for biotinylated19 DNA probes), pre-hybridization and some probe purification steps [17]; thus rapid. It also enables 100 folds increase in the binding of single-stranded DNA to filter by treatment with alkali and steam [17,18,20,22] starting from cell lysis to DNA denaturation and binding to the membrane. The process of DNA binding by alkali and steam, is also rapid (8 min) [17,18,19,20,22] and dispenses the need for 2 h vacuum baking as in nitrocellulose membrane or expensive UV cross-

linking which is required for the nylon membranes. This makes the filter more useful with respect to sensitivity, specificity and rapidity of detection22 besides being economic.

The Whatman 541 filter paper is a supreme quantitative filter paper featuring high wet strength and chemical resistance. These papers are acid hardened which reduces ash to an extremely low level thereby making it more suitable for use in research [21].

Efficient binding up to 100 fold more [17,18,22] of single stranded DNA is achieved by touching the plated colonies or spotting broth cultured cells on the M/S Whatman 541 filter paper and placing it on a 0.5 N NaOH and 1.5 M NaCl (lysing solution) pre- soaked 2 number of blotting papers under steam from a syringe sterilizer or on a double-decker steaming water bath (Figure 7) for three minutes and then placing it on a freshly pre- soaked 2 blotting papers in fresh lysing solution for one min. The alkali treatment and exposure to steam cause rapid lysis of cells, denaturation and binding of single-stranded DNA on to the filter simultaneously [17,18,19,20,22]. This is followed by soaking the filter with blotting paper and immersing it in neutralization solution (1.0 M Tris-HCl [pH 7.0] and 2.0 M NaCl) for 4 minutes. The air or IR dried filter paper is put directly for brief hybridization for up to 30 minutes (in view of 100 times more DNA binding) and use of abundant oligo or polynucleotide DNA probe. No pre-hybridization is required [17,18,19,23,24]. A brief post-hybridization washing was followed, and interpretation of result made by the florescence emitted under UV torch or as per the probe label used.

It has been seen that many components like fat free milk powder, dextran sulphate, Denhardt's solution are not essential and can easily be replaced with less costly, less complicated and more effective ingredients which increase sensitivity, reduces background and cost and easier for trouble-shooting. These even can be dispensed while using the Whatman 541 filter paper. Hybridization can be performed with the DNA probe in any standard hybridization solution like in 4 x SET buffer (lx SET buffer is 0.15 M NaCl, 0.03 M Tris hydrochloride [pH 8.0] and 1 mM EDTA), 0.5% sodium dodecyl sulfate (SDS), 24 g of heat-denatured salmon sperm DNA per ml. Alternatively, hybridization can be performed in 10% polyethylene glycol, 1% crystalline-grade bovine serum albumin,1 mM Na_2 EDTA and 7% SDS in 0.5 M $NaHPO_4$ (pH-7.2) buffer as hybridization solution.

Figure 7: Double decker steaming water bath developed in the laboratory

Interpretation of hybridization result is made rapid by the checking fluorescence under UV torch/ lamp for the dye like DAPI (4′,6-diamidino-2-phenylindole) or 7-AAD (7-aminoactinomycin D) or Hoechst 33258 (33342, 34580) or DNA binding fluorescent quantum dots etc.

The process can be completed with accurate results in less than one hour and several samples can be tested simultaneously at the same time and cost. This is most ideal for epidemiological studies during CIVID-19 times. Rapid hybridization also helps in reducing false positive results because of the dispensation of prolonged contact of the probe with filter, DNA template, and re-association of polynucleotide probe during hybridization.

DNA bound Whatman 541 filter papers from Field test samples can also be preserved or safely transported for testing in Reference laboratories.

Easy, cheap and rapid isolation of DNA.

After the first successful identification of DNA by Friedrich Miescher during 1860s, nucleic acids are now central to obtaining any biological information. Several modifications in isolation from phenol-chloroform extractions to column chromatography as well as automated methods and "lab-on-chip" technology have been developed after the original 1869 methods [25] developed earlier. In spite of the efforts made, a simple and effective extraction method is still a major challenge in view of its widespread downstream applications like the point-of-care diagnostics (POC-Dx) [25]. The ubiquitous nature of DNAase that we have discussed earlier [1-7] at Sl. No. 1 and its heat resistant forms [26], present as a contaminant in the commercial preparations of pancreatic RNase, are often a major problem. Widespread quest is on for an efficient extraction method that can be integrated in a POC-Dx system [25] and other applications.

Pancreatic RNase preparations are the largest source [4,5] of commercial RNase. Inactivation of contaminating DNase activity in pancreatic RNase preparations is widely accepted [3,4,5,6,7,26] as 'often a problem'. This problem has been encountered in many batches/ lots of pancreatic RNase preparations that are available commercially. Thus, various protocols but preferably boiling for 10 min to 30 min followed by slow cooling [5,6,26] and even with repeated boiling for 30 min 25 at much lower RNase concentrations, have been proposed by the manufacturer of commercial RNAase. However, we have seen that nothing works in some lots of RNAase preparations that are available commercially [5,6,26]. The proposed method is an efficient and simple solution to the problem.

We have used the same salts as used for DNA immortalization (Sl. No.1), but at the following concentrations for easy, rapid and quantitative isolation of DNA [5,6,7] from E. coli.

Sodium chloride (NaCl)- 0.1 M

Trisodium citrate ($Na_3C_6H_5O_7$)-0.06 M

P^H = 7.0 to 7.2

It has been seen that the above buffer selectively permits action of the enzymes required for DNA isolation (lysozyme, proteases and ribonuclease) but typically inhibits the DNA degrading enzymes (deoxyribonuclease-1, Restriction endonucleases, S1 nuclease etc.). There is no need for heat inactivation of contaminating DNase or other enzymes, when the DNA is in the above buffer. We have used up to 200 µg/ml of pancreatic RNase without heat treatment (Figure 8) and successfully tested even up to 10 mg/ml concentration of RNase powder applied directly for selective degradation of RNA. Direct

additions of pancreatic RNase powder to DNA and RNA in the above solution selectively degrade the RNA without affecting the DNA.

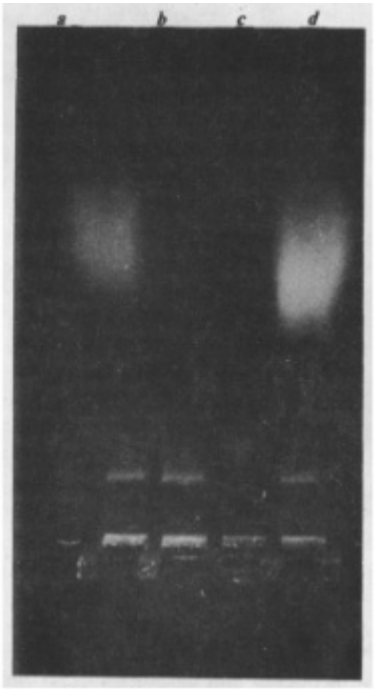

Figure 8: Pancreatic RNase powder added directly to the DNA and RNA pellet dissolved in the new buffer (lane 'a' and 'b') and in conventional TE buffer (lane 'c' and 'd'). Agarose gel electrophoresis revealed the presence of only DNA but no RNA (lane b) in the new buffer, whereas loss of both DNA and RNAin the conventional buffer (lane c). Lane 'a' and 'd' are the two buffer controls.

Conclusion

Although we have also got good results with 0.05 M sodium citrate during the isolation of DNA, we recommend a concentration of 0.06 M sodium citrate in view of the discussion in the section-1.

DNA (after degradation of RNA) present in 0.1 M NaCl after phenol-chloroform processing, can be precipitated directly by addition of ethanol, dispensing the need for additional supplementation of sodium/potassium/ammonium acetate or lithium chloride.

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