WELCOME



Venue

Institute of Endemic Diseases - University of Khartoum

Medical Campus

www. tropmedicine.org

TIBA Sudan | Advance Malaria Course November 2018

TIBA

Tackling Infections to Benefit Africa (TIBA) is an Africa-led, wide-ranging, multi-disciplinary research programme that explores and draws lessons from the ways that different African health systems tackle infectious diseases. TIBA will help empower African scientists to effectively and sustainably tackle neglected tropical diseases (such as schistosomiasis, malaria, trypanosomiasis and lymphatic filariasis), and improve preparedness for epidemics (such as Ebola). Tiba means "to cure an infection" in Swahili.

TIBA Aims

1. Improve the diagnosis and surveillance of infectious diseases in resource-poor settings
2. Improve the deployment of existing drug treatments and enhance local capacity to develop new ones
3. Improve the deployment of existing vaccines and enhance local capacity to develop new ones
4. Improve the management of endemic and epidemic infectious diseases by:
    1. Strengthening health systems, governance and ethics
    2. Improving policy development and implementation
    3. Enhancing capacity to respond to infectious disease emergencies

TIBA Sudna

The TIBA-Sudan Rapid Impact Project aims to contribute to Malaria elimination from Khartoum State: Improvement of the health system and detection of persistent transmission foci of Plasmodium species. It is partnership between the Institute of Endemic Diseases, The Federal ministry of Health, Sudan, The directorate of laboratories, Khartoum State Ministry of Health and Edinburgh University. Part of the capacity building objective, the institute of Endemic Diseases is organizing an advance malaria training course for junior scientist, health staff and post graduate students working on malaria. The course will be facilitated by local expert and international malariologist and will include both theoretical lectures and hands on practical's

Training Course Organizers



Professor Maowia Mukhtar

Immunologist with extensive research experience.
Long teaching experience of basic and advance immunology courses.
Experience in research capacity building.
Research on leishmaniasis taxonomy, diagnostics and vaccines.
Research on TB drug resistance and diagnostics.
Malaria immunity, drug resistance and parasite diversity.
Atlas GIS mapping.
E.mail: mmukhtar@iend.org



Sara Ibrahim A.Latif

M.Sc. Molecular Medicine
PhD student-Molecular Biology-
Institute of Endemic Diseases-University of Khartoum
Good experience in medical research, molecular biology and immunology.
Email: sursur555@gmail.com
Email: Sara_A.Latif@tropmedicine.org

Workshop Program



Sunday

Day One 25 - 11 - 2018

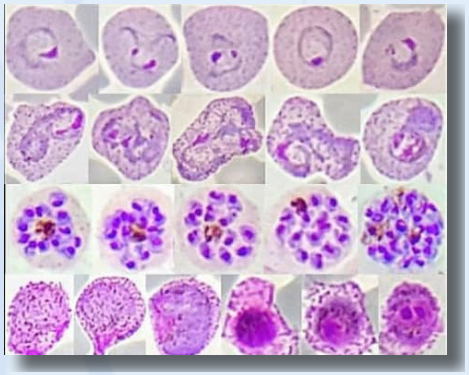
Time	Topic	Facilitator
8:30 - 9:30	**Registration**	
9:30 - 10:00	Opening remarks	
10:00 - 10:30	Tea Break	Tea Break
10:30 - 11:20	Clinical Malaria	Professor Ahmed M Musa
11:20 - 12:10	Malaria Epidemiology	Professor Takafira Mduluza
12:10 - 12:55	Lunch break	Lunch break
12:55 - 1:45	Vivax malaria as emerging diseases	Dr Mozamil Mahdi
1:45 - 2:00	Practical groups formation	
2:00 - 3:45	Group (١) Malaria culture Group(٢) DNA extraction with Kits	Dr Zeinb Imam Mrs Einas Awad
3:45 - 4:00	Tea Break	Tea Break
4:00 - 5:45	Group (٢) Malaria culture Group(١) DNA extraction with Kits	Dr Zeinb Imam Mrs Einas Awad
5:45 - 6:00	Closing of day one	



Monday

Day Two 26 - 11 - 2018

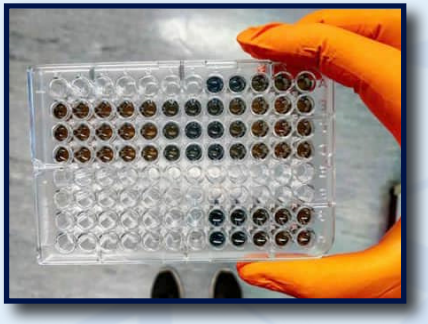
Time	Topic	Facilitator
9:00 - 10:00	Malaria Biology	Professor David Arnot
10:00 - 10:15	Tea Break	Tea Break
10:15 - 11:15	Malaria vaccine	Dr Samsom Kinyanjui
11:15 - 12:30	Group (١) PCR +RLFP Group(٢) Malaria culture harvesting and staining	Ms Manasik Elghali Dr Zeineb Imam
12:30 - 1:15	Lunch break	Lunch break
1:15 - 2:45	Group (١) PCR +RLFP Group(٢) Malaria culture harvesting and staining	Ms Manasik Elghali Dr Zeineb Imam
2:45 - 3:00	Tea Break	Tea Break
3:00 - 5:45	Group (٢) PCR +RLFP Group(١) Malaria culture harvesting and staining	Ms Manasik Elghali Dr Zeineb Imam
5:45 - 6:00	Closing of day two	



Tuesday

Day Three 27 - 11 - 2018

Time	Topic	Facilitator
9:00 - 10:00	Malaria pathogeneses	Professor Eltahir Awad Gssim
10:00 - 10:15	Tea Break	Tea Break
10:15 - 11:15	Malaria Immunology	Professor Takafira Mduluza
11:15 - 12:30	**Group (1) PCR +RLFP** **Group(2) Malaria culture harvesting and staining**	Ms Manasik Elghali Dr Zeineb Imam
12:30 - 1:15	Lunch break	Lunch break
1:15 - 2:45	Group (1) Gel electrophoresis Group(2) RT PCR	Mrs Einas Awad Dr Zeineb Imam
2:45 - 3:00	Tea Break	Tea Break
3:00 - 5:45	Group (2) Gel electrophoresis Group(1) RT PCR	Ms Manasik Elghali Dr Zeineb Imam
5:45 - 6:00	Closing of day two	



Wednesday

Day Four 28 - 11 - 2018

Time	Topic	Facilitator
9:00 - 10:00	**Molecular and serological diagnosis of malaria**	**Professor David Arnot**
10:00 - 10:15	Tea Break	Tea Break
10:15 - 11:15	Malaria control	Dr. Faiza Mohamed Osman
11:15 - 12:30	**Group (1) ELISA serology for Malaria Ag** **Group(2) ELISA cytokines analysis (coating Ab)** **Group (3) Whole blood stimulation**	Mr Salah Marajan Dr Mona Omer Ms. Sara A.Latif
12:30 - 1:15	Lunch break	Lunch break
1:15 - 2:45	Group (2) ELISA serology for Malaria Ag Group(3) ELISA cytokines analysis (coating Ab) Group (1) Whole blood stimulation	Mr Salah Marajan Dr Mona Omer Ms Sara A.Latif
2:45 - 3:00	Tea Break	Tea Break
3:00 - 5:45	Group (3) ELISA serology for Malaria Ag Group(1) ELISA cytokines analysis (coating Ab) Group (2) Whole blood stimulation	Mr Salah Marajan Dr Mona Omer Ms. Sara A.Latif
5:45 - 6:00	Closing of day two	



Thursday

Day Five 29 - 11 - 2018

Time	Topic	Facilitator
9:00 - 10:00	**Malaria Vector**	**Dr Tellal Babkir**
10:00 - 10:15	Tea Break	Tea Break
10:15 - 11:15	Malaria Genome & Primer design for malaria research	Dr Sahar Bakhit
11:15 - 12:30	**Group (1) ELISA IL6 cytokines** **Group(2) ELISA TNF-α** **Group (1 or 2) Whole blood stimulation harvesting**	Mr Salah Marajan Dr Mona Omer Ms. Sara A.Latif
12:30 - 1:15	Lunch break & Certificate awards	Lunch break & Certificate awards
1:15 - 2:45	Group (1) ELISA IL6 cytokines Group(2) ELISA TNF-α Group (1 or2) Whole blood stimulation harvesting	Mr Salah Marajan Dr Mona Omer Ms. Sara A.Latif
2:45 - 3:00	Tea Break	Tea Break
3:00 - 5:45	Group (1) ELISA IL6 cytokines Group(2) ELISA TNF-α	Mr Salah Marajan Dr Mona Omer
5:45 - 6:00	Closing of day two	

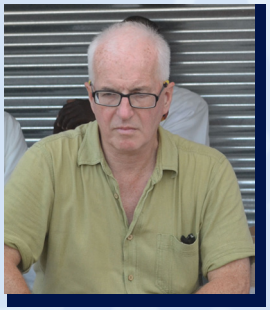


Professor Takafira Mduluza

Professor: Biochemistry & Immunology, University of Zimbabwe
Director for Immunology & Infection Research
Honorary Professor, University of KwaZulu Natal, South Africa
Fellow, Zimbabwe Academy of Science, Researcher TIBA_Zimbabwe
Honorary Visiting Research Fellow, Tulane University School of Public Health.

Professor Takafira Mduluza is based in the Biochemistry Department, University of Zimbabwe as Professor of Biochemistry and Immunology. He is a Biomedical Scientist specializing in the Biochemistry and Immunology of Infectious Diseases including parasitic infections. His work concentrates on the biochemistry and immunological consequences of infectious diseases in endemic populations and immuno-epidemiological studies of the relationship between defined immune responses and acquisition of clinically protective immunity.

Email mduluza@medic.uz.ac.zw , tmduluza@yahoo.com



Professor David Arnot

Professor at University of Edinburgh, UK
Zhejiang University, China
Works on malaria antigenic variation, the molecular cell biology and pathology of Plasmodium falciparum and teaches and supervises research in various aspects of vaccinology and biotechnology. An amateur interest in the genetics of hazel trees, hazel nut farming and wildwood reforestation in Scotland.

davidarnot@intl.zju.edu.cn

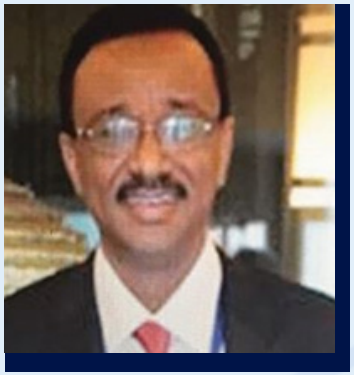


Dr. Sam Kinyanjui

is the Head of Training and Capacity Building at the KEMRI-Wellcome Trust Programme in Kenya and the Director for the Initiative to Develop African Research Leaders (IDeAL). Prior to current position he pent 16 years doing research on the immunology and molecular biology of malaria parasites. During this period he developed a strong interest in capacity building for health research in Africa

Dr. Kinyanjui is part a team at KWTRP that has developed the capacity to run controlled human malaria infection (CHMI) studies in Kilifi, Kenya and are using this framework to explore the mechanisms underlying immunity. Part of the work imbedded with these studies are funded through TIBA

skmuchina@kemri-wellcome.org



Professor Ahmed Mudawi Musa

Professor of immunology and infectious diseases, Institute of Endemic Diseases, University of Khartoum.

Prof Ahmed Mudawi Musa (MBBS, DTM&H, MSc TM&IH, MRCPI, Ph D) is a professor of immunology and infectious diseases at the Institute of Endemic Diseases. He is a physician specialist in internal and tropical medicine and clinical infectious diseases. He joined the Institute of Endemic Diseases in 2000. Prof Musa has cumulative experience in infectious diseases especially leishmaniasis. He is now giving specialist consultations in the diagnosis and management of various infectious diseases in Sudan.

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Dr. Muzamil Mahdi Abdel Hamid

Associate Professor of Immunology and Molecular Parasitology ,Institute of Endemic Diseases, University of Khartoum.

Head Department of Parasitology and Medical Entomology.

Major research areas are molecular and immunology of malaria parasites. Molecular epidemiology of plasmodium vivax in relation to Duffy negative transmission, Epidemiology and control of parasite and Arbor viral vectors, insecticide resistance in mosquito vectors, Arboviruses vectors.

mahdi@iend.org



Professor Eltahir Awad Gasim Khalil

Professor at University of Khartoum

Experienced Professor with a demonstrated history of working in the education management industry. Skilled in Research, Clinical Research, Epidemiology, Teaching, and Higher Education. Strong education professional with a MBBS, LRCP, MRCS, FRCPath focused in Surgery and Medicine from University of Khartoum.

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Dr. Faiza Mohamed Osman

Head of department of epidemiology and nutrition at Institute of Endemic Diseases University of Khartoum.

Dr. Faiza M Osman is an expert Assistant Professor in medical research ethics, and nutrition

E.mail. faizaosman@iend.org

Local Facilitators



Dr. Tellal Babiker Ageep

Department of Epidemiology, Tropical Medicine Research Institute, national Center for research, Ministry of High Education and Scientific Research, Khartoum Sudan.

Major research areas are identification of environmental envelopes of vectors, vector habitat and Distribution mapping/stratification, analysis of vectors/disease spatial patterns, Disease risk mapping/ stratification/prediction, and direction of control efforts.

Expert on Epidemiology and Biostatistics. Expert on application of space-related technologies; including remote sensing, GIS and GPS for planning, environmental Management / assessment and studies of vectors of diseases.

tellalageep92@gmail.com



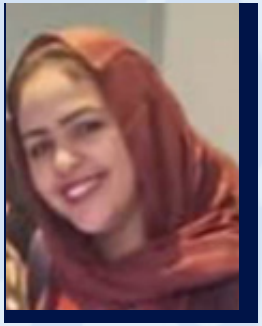
Dr. Sahar Mubarak Bakheit

Head Department of Molecular Biology- Institute of Endemic Diseases- University of Khartoum

Assistant Professor of Molecular biology and she is an expert in medical research and infectious diseases.

E.mail. saharbakheit@iend.org

Technical Staff



Dr. Zeinab S. Imam

Postdoctoral Researcher at Institute of Endemic Diseases

PhD. In Molecular Biology and Parasitology

Good experience in molecular biology techniques DNA and RNA extraction, amplifying nucleic acid by using PCR, real time PCR, Gel Electrophoresis, and RFLP- PCR) at Molecular biology Lab.

Zeinabimam@hotmail.com



Mrs. Einas Awad Ibrahim Osman

PhD student at Institute of Endemic Diseases

M.Sc. Medical Laboratory Sciences (Microbiology)

Good experience in molecular microbiology, clinical and research laboratories.

Technical Staff



Ms. Manasik Elghali Hamed Abdalrazig

PhD student at Institute of Endemic Diseases
M.Sc. of immunology
Good experience in Molecular Biology and Immunology techniques
manasikghali@gmail.com



Mr. Salah Abdalla Boshara Marajan

PhD student at Institute of Endemic Diseases
M.Sc. of immunology
Good Experience in Molecular Biology and Immunology researches, high experience in data analysis.



Dr. Mona Omer Ahmed Awad Elkarim

Postdoctoral Researcher at Institute of Endemic Diseases
PhD. In Biomedical Sciences –Microbiology
Good experience in Molecular Biology and Immunology researches.

Practicals

Cultivation of Plasmodium falciparum

Materials and Reagents:

- RPMI (Roswell Park Memorial Institute) 1640 medium (biowest, cat N L0495500-) or (Sigma, USA) R 6504.
- Uninfected erythrocytes (blood group O).
- Sodium bicarbonate.
- Albumax.
- Gentamycin (40mg/ml).
- Phosphate buffer solution (PBS).
- Giemsa stain and Methanol.



Equipment:

- Co2 incubator (37 °C).
- 25ml Tissue Culture Flask (Biofil).
- Centrifuge.
- Sterile pipettes.
- Sterile falcon tubes.
- Glass slides.
- Microscope, fluorescence.
- Filtered tips (1ml).
- Syringe filter (0.22µm).

Procedure

Preparation of Sample:

- Collect Five ml of parasited blood samples in Citrate Phosphate Dextrose
- Adenine (CPDA-1) tube from confirmed malaria patients.
- Calculate the parasitaemia of all samples by the equation
- Number of observed asexual parasites x 100 ---------- = parasites of blood in percentage% total n. of RBC scanned in 25 microscopic fields.
- The parasitaemia must range between 0.4- 0.6%.

Preparation of Media:

Prepare two hundred and fifty milliliters as follows

- 4.05g of RPMI 1640.
- 0.5g Sodium bicarbonate.
- 1.2g Albumax.
- 1ml Gentamycin (40mg/ml).
- Adjust the pH to 7.2 and filter through 0.22 µm filter.

Preparation Washing Medium:

Phosphate buffer solution PBS is used as a washing medium.

- Add 500ml to one tablet of PBS (Gibco™ 18912014)
- Adjust the pH using pH Meter to 7.4 then sterilize by
- autoclaving and store at 15°- 30°c in a dark place

Preparation of Erythrocytes (RBCs):

- Collect ten ml of blood from 0+ve blood group healthy subject.
- Centrifuge at 2000 rpm for 10 minutes.
- Discharge the upper phase which contained the white blood cells.
- Wash the blood three times with sterile PBS buffer (10 minutes at 2000 rpm).
- Add equal volume of incomplete media (RPMI media without Albumax) to the washed red blood cells (the pellet) to make 50% hematocrit and stored at 4°C.

Initiation of the culture:-

- Warm complete medium to 37° C in water-bath or heater block.
- In 25mlTissue Culture Flask (Biofil), add 4.75 mL of complete medium.
- Add packed uninfected erythrocytes to packed infected erythrocytes as to
- obtain a total volume of 250 µL.
- Add this volume to the medium to obtain a 5% hematocrit.
- Stir gently.
- Maintain the culture of P. falciparum in incubator in the following conditions:
  - Temperature: 37° C.
  - Gas mixture: 5% CO_2, 5–10% O_2 and 85–90% N_2.
  - Humidity: > 90%.

Checking of the Culture growth

After 24 Hours:

- Check the culture media by making thin and thick blood films stained with Giemsa and examine under microscope to compare the current parasitaemia with the initial parasitaemia.
- Remove the media using sterile Pasture pipette.
- Add fresh complete media and fresh RBCs 50% and mix probably.

DNA Extraction with Kits (Blood Protocol)



Materials and reagents:

- Intron Kits.
- Eppendorf tube.
- Microcentrifuge tube.
- Proteinase K.
- PBS.
- Ethanol.

Equipment:

- Water bath.
- Centrifuge.

Procedure:

1. Pipet 200 µl of whole blood or body fluids into a 1.5 ml Eppendorf tube.
2. Wash by PBS.
3. Add 20 µl of Proteinase K and 5 µl of RNase A Solution into sample tube and gently mix.
4. Add 200 µl of Buffer BL into upper sample tube and mix thoroughly.
5. Incubate the lysate at 56°C for 10 min (Water bath) .
6. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the rid.
7. Add 200 µl of absolute ethanol into the lysate, and mix well by gently inverting 5 - 6 times or by pipetting. DO NOT vortex. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside of the lid.
8. Carefully apply the mixture from step 6 to the Spin Column (in a 2 ml Collection Tube) without wetting the rim, close the cap, and centrifuge at 13,000 rpm for 1 min. Discard the filtrate and place the Spin Column in a 2 ml Collection Tube (reuse).
9. Add 700 µl of Buffer WA to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and reuse the Collection Tube.
10. Add 700 µl of Buffer WB to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and place the Column into a 2.0 ml Collection Tube (reuse), Then again centrifuge for additionally 1 min to dry the membrane. Discard the flow-through and Collection Tube altogether.
11. Place the Spin Column into a new 1.5 ml tube, and 30 - 100 µl of Buffer CE directly onto the membrane. Incubate for 1 min at room temperature and then centrifuge for 1 min at 13,000 rpm to elute.

Polymerase Chain Reaction (PCR)



Background:

The Polymerase Chain Reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Allele-specific polymerase chain reaction (AS-PCR), also known as amplification refractory mutation system (ARMS) or PCR amplification of specific alleles (PASA) is a PCR-based method which can be employed to detect the known SNPs .The concept of AS-PCR was initiated by Newton et al, approximately six years after PCR was invented. In this approach, the specific primers are designed to permit amplification by DNA polymerase only if the nucleotide at the 3'-end of the primer perfectly complements the base at the variant or wild-type sequences. After the PCR and electrophoresis, the patterns of specific PCR products permit the differentiation of the SNPs. Several innovative approaches have been employed to detect the presence of specific PCR product. Some are based on probe hybridization which requires specific labeled probes and melting curve analysis which requires nucleic acid stains.AS-PCR has been utilized widely in many areas of study such as pharmacogenetics, genetic disorders microbiology and others.

Reaction Requirements:

PCR are as simple as adding primers and template to an aliquot of our PCR master mix . It contains:

Component	Concentration
10x Buffer	10X
dNTPs mix	2.5mM each
Taq DNA Polymerase	2.5 U
Gel loading buffer	

The 10X PCR buffer contains: 500 mM KCL, 100mM Tris-HCL (PH 8.3), 15 Mm Mgcl the final concentration of this ingredients in PCR mix are: 50Mm KCL, 10 Mm.
Tris-HCL,1.5 Mm Mgcl .

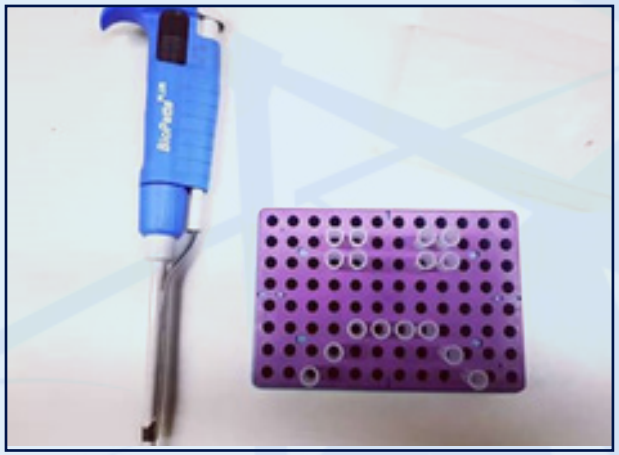
Procedure:

- Briefly mix all reagents before beginning the procedure using the vortex.
- Add template and primers into master mix tube.
- Add distilled water into tube to a total volume of 20µl or 50 µl do not calculate the dried components.
- Place your tube into a thermo cycler and start PCR.

Note: Time differs from Protocol to another:

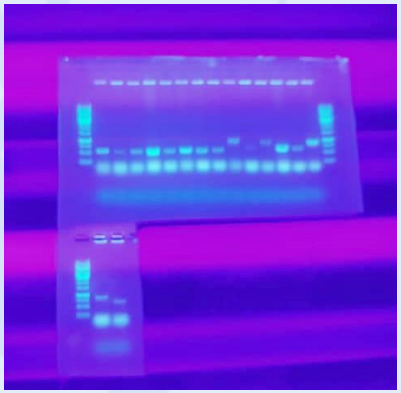
- PCR is very sensitive to contamination from outside condition, So you should reduce the chance of contamination by wearing gloves using autoclaved tips and PCR tube.
- Many things must be placed in the ice like Taq DNA polymerase.

Restriction Fragment Length Polymorphism (RFLP):



- After PCR amplification of the genes the following must be added:
  - Restriction enzymes 0.5 microlitre
  - Restriction buffer 1.5 microlitre
  - PCR Product 8 microlitre
- The mixture must be mixed well and incubate for 55oC for 4 hours to inactivate the enzyme. If there is a restriction enzyme site, the endonuclease cuts the PCR product.
- The different alleles will be easily differentiated by electrophoresis through 3% or 4% agarose gel stained with ethidium bromide at 100v for 40min and visualized by UV light.

Agarose Gel Electrophoresis(RFLP):



Background:

Gel electrophoresis is the standard lab procedure for separating DNA by size (e.g., length in base pairs) for visualization and purification. Electrophoresis uses an electrical field to move the negatively charged DNA through an agarose gel matrix toward a positive electrode. Shorter DNA fragments migrate through the gel more quickly than longer ones. Thus, you can determine the approximate length of a DNA fragment by running it on an agarose gel alongside a DNA ladder (a collection of DNA fragments of known lengths).

Regents:

- TBE.
- Tris-base: 242 g.
- Acetate (100% acetic acid): 57.1 ml.
- EDTA: 100 ml 0.5M sodium EDTA.
- Add dH2O up to one litre.
- Agarose Powder.
- Ethidum bromide (stock concentration of 10 mg/mL).

Equipment:

- Casting tray.
- Well combs.
- Voltage source.
- Gel box.
- UV light source.
- Microwave.

Procedure for 1.5% Agarose Gel:

- Measure 1.5 g of agarose.
- Mix agarose powder with 100 mL 1xTBE in a microwavable flask.
- Microwave for 13- min until the agarose is completely dissolved (but do not over boil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up).
- Let agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask), about 5 mins.
- Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 23- µl of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light.

After completion of PCR cycles, the product was subjected to electrophoresis in a 1.5% agarose gel and tested by transferred of 7 µL of PCR product to gel walls. The visualization was obtained by adding of 4 µL of ethidum bromide to the pre-melted agarose gel. Also positive and negative control was loaded in first two wells in each run. Electrophoresis was carried out at 80 volts for 45 minutes. For visualization of the PCR agarose gel using ultraviolet transilluminato and Polaroid picture was taken and documented.

Quantitative Real time-PCR assays RT.PCR:

A real-time quantitative PCR assay is used to estimate the relative quantification of the genes. This will be done by using 2x Real Mod™ Green Real Time PCR Master Mix kit (iNtRON biotechnology / catalog No. 25344), that contained Eva Green dye master mix for 500 reactions and QIAGEN›s real-time PCR cycler, the Rotor-Gene Q (11127).

Equipment and Reagents:



- DNA or cDNA.
- PCR Master Mix.
- Primers (Forward and Reverse).
- RNase free water or DEPC (Diethylpyrocarbonate).
- Micropipettes and filtered tips.
- 1.5 ml micro centrifuge tubes.
- Vortex and Spinner.
- 36-Well Rotor (red in color).

Procedure:

- Prepare PCR mix in a total reaction volume of 20 µl including template DNA.
  - PCR Master mix 10 µl.
  - Forward primer 2 µl.
  - Reverse primer 2 µl.
  - Template 2µl.
  - Distilled water 4µl.
- The PCR mix must gently mix by inversion, Spin to reduce bubbles.
- Place Real-time PCR tubes in the Real-time PCR instrument and start the cycling program.
- Record the Ct (threshold) after the reaction complete and use the standard curve to determine the efficacy of the real-time PCR: E = 10(-1/slope) -1.

Lymphocytes Stimulation in a Whole Blood Sample



Introduction:

Heparinized venous blood samples were diluted 1:1 with PBS in a culture plate and stimulus antigen added , incubation for 48 hours then collection of plasma should take place.

Reagents: [PBS]

Solution preparation:

- One tablet of PBS.
- 100 ml of DW.

Equipment:

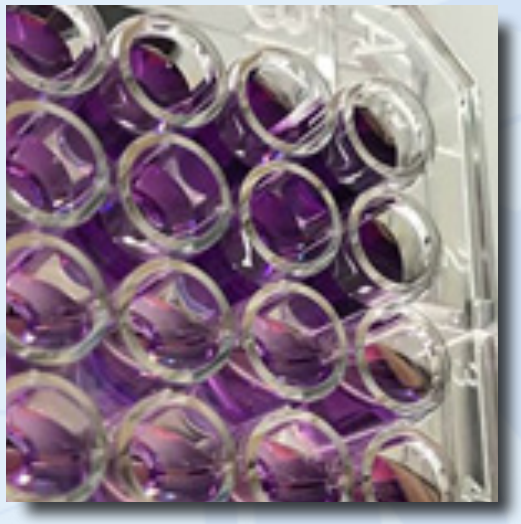
- Biological Safety Cabinet.
- Falcon tubes 50 ml .
- Culture plate.
- Minus 80°C or minus 20°C freezer.
- Cryo vials.
- Pipette and tips blue ,yellow tips.
- Eppendorf.
- Mini centrifuge.
- 100 micro L pipette.

Sample Collection: 2ml of venous blood collected within heparinized tube under sterile condition , mixed gently and kept within 4 °C.

Sample Processing in Lab:

- Autoclaving of PBS, yellow tips, blue tips and eppendorf.
- Open the safety cabinet make sure it is sterile.
- Bring all reagents and equipment to safety cabinet.
- Mix equal volume of blood and PBS 1:1.
- Label the culture plate and distribute samples within wells.
- Insert desired antigen within one well leaving other well of the same sample as control.
- Incubate the culture plate in CO2 incubator at 37°C for 48 hours.
- Then empty each well within labeled eppendorf tube.
- Centrifugation for 10 min at speed 1500.
- Collect the supernatant into cryo vials for storage.
- Store ate -80 or -20 °C.

ELISA-Based Identification and Detection of Microbes



Introduction:

Enzyme-Linked Immunosorbent Assay (ELISA) is a highly sensitive immunological technique with high specificity for the detection of particular antigen (Ag) or antibody (Ab) in a sample using enzyme-linked antibodies.

An ELISA is a five-step procedure:

- Coat the microtiter plate wells with antigen.
- Block all unbound sites to prevent false-positive results.
- Add antibody to the wells.
- Add anti-antibody conjugated to an enzyme; and.
- Reaction of a substrate with the enzyme to produce a colored product, thus indicating a positive reaction.

Principle:

The basic principle of ELISA is to use an enzyme attached to antibody to detect either antigen or antibody in an immunoassay to allow quantification through the development of color after the addition of a suitable chromogenic substrate. ELISA is used to target the antigen with high specific antibodies which are conjugated to the enzymes. These enzyme-conjugated antibodies are allowed to bind to the respective antigens, and then after the addition of the suitable chromogenic substrate to this (Ag–Ab–E complex), color will be produced due to the enzymatic activity. The enzymatic reaction stopped and the color developed will be detected spectrophotometrically by ELISA reader.

Types of ELISA

- Direct ELISA.
- Indirect ELISA.
- Sandwich ELISA.
- Competitive ELISA.
- Multiplex ELISA.

Note:

Antigens, serum samples, and assay chemicals used in ELISA may be potentially infectious and/or toxic. Wear gloves and lab coat before handling all serum and plasma specimens of blood–borne infections.

Materials:

- 96-Well Microtiter Plates.
- ELISA Reader.
- Microplate shaker.
- Multichannel pipettor.
- Micrpipetts and Eppendorf Tubes.
- Yellow tips.
- White tips.
- Blue tips.

Buffers:

Coating Buffer (0.1 M Carbonate bicarbonate buffer, pH 9.6):

(a) 3.03 g Na2CO3.

(b) 6.0 g NaHCO3.

(c) 1,000 ml distilled water.

(d) Store at 4 _C.

Phosphate buffer Saline (PBS), pH 7.4: 1l

(a) 2.32 g Na2HPO4.

(b) 0.2 g KCl.

(c) 0.2 g K3PO4.

(d) 8.0 g NaCl (500 ml distilled water).

(e) Store at 4 _C.

Blocking buffer solution:

(a) 1,000 ml PBS buffer.

(b) 0.5 ml Tween 20.

(c) 10 g BSA.

(d) Store at 4 _C.

(e) (1 % BSA, serum, nonfat dry milk, casein, gelatin in PBS).

Washing solution:

(a) [PBS or Tris-buffered saline (pH 7.4) with detergent such as 0.05 % (v/v) Tween 20].

(b) 0.5 ml Tween 20.

(c) 999.5 ml PBS buffer.

(d) Store at 4 _C.

Antibody dilution buffer:

Primary and secondary antibody should be diluted in blocking solution.

Reagents:

Purified antigens/antibodies, Enzyme-conjugated (alkaline phosphatase, Horseradish peroxidase) antibodies (Primary/Secondary), PNPP (p-Nitrophenyl phosphate, Disodium salt), ABTS (2,20-Azinobis [3-ethyl benzothiazoline-6-sulfonic acid]- diammonium salt), OPD (O phenylenediamine dihydrochloride), TMB (3,30,5,50-tetramethyl benzidine), Hydrogen peroxide, Tween 20, and above-mentioned chemicals for the preparation of buffers.

Sandwich ELISA

Test procedure:

Coating:

1. Dilute the antibody with coating buffer and coat wells of ELISA plate 100 ml/well.
2. Cover the plate with plastic and incubate 2 h at room temperature or overnight at 4 C.
3. Remove the antibody solution from the wells of plate by flicking the plate over sink and wash the plate thrice by filling the wells with 300 ml of washing buffer/PBS.

Blocking

1. Block the nonspecific protein binding sites in the coated wells by adding 100 ml blocking buffer/5 % serum in PBS/well.
2. Cover the plate with plastic and incubate at 4 _C overnight or 2 h at room temperature.
3. Remove the blocking buffer from the wells by flicking the plate over sink and wash the plate thrice with PBS.

Standard and Samples Incubation

1. Dilute the standards/samples with blocking buffer and transfer 100 ml to each well.

2. Cover the plate with plastic and incubate at 4 _C overnight or 2 h at room temperature.
3. Remove the standard/samples solution from the wells by flicking the plate over sink and wash the plate thrice with PBS.

Incubation with HRP-Conjugated Antibody

1. Dilute the labeled antibody (0.25–2 mg/ml) in blocking buffer and add 100 ml in each well.
2. Cover the plate with plastic and incubate for 1 h at room temperature.
3. Remove the blocking buffer from the wells by flicking the plate over sink and wash the plate thrice with PBS.

Detection

1. Add 100 ml or 50 ml of substrate (for ALP or HRP accordingly) solution per well.
2. After sufficient color development, add 50 ml of stopping buffer (8N H2SO4).
3. Read the absorbance of each well at 492 nm with a Microplate reader.

Interpretation of the results

Prepare a standard curve from the data produced from the serial dilutions with concentrations on the X-axis vs. absorbance on the Y-axis. Interpolate the concentrations of the sample from this standard curve. Be consistent when adding standards to the assay plate.

Add standards first and then samples to calibrate the results.

Add standards to plate in the order from low concentration to high concentration as this will minimize the risk of compromising the standard curve.

Results of ELISA can be measured both qualitatively and quantitatively; the qualitative results provide a simple positive or negative result in the form of a presence or absence of a visible colored product for a sample, whereas in quantitative ELISA, the optical density (or fluorescence) is measured and plotted into a standard curve, which is typically a serial dilution of the protein to be quantified.