Setting up a Diagnostic Molecular Laboratory

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Why go Molecular?

- Impressive growth and developments in Molecular Diagnostics – last 15 years.
- Advantages of molecular diagnostics
  - Quicker turn around time
  - Improved sensitivities
  - Increased accuracy
  - Marked cost savings
- Industry driven by technology

A growing field

- Worldwide MDx market – around $5.8 billion!
- Steady growth being fuelled by:
  - New technologies
  - Innovations
  - Expanded test applications
- Very broad Market :
  - Not limited to one field of study – genetics, infectious diseases, oncology, haematology and pharmacology

Designing a lab

- Create a successful workflow for PCR
  - Earlier years – contamination of PCR rxn,s with amplification product from previous PCR was a potential problem
- To Combat contamination –
  - Three separate rooms
    - Preparing the reaction
    - Amplification
    - Analysis of amplified products
- New instruments – closed systems – less contamination risk
- Facilities still need to be well designed
- No inflexible guidelines in molecular lab design – primary emphasis is avoidance of contamination with each step of the workflow

Important components of setting up a quality laboratory

- GCLP – ensure that quality policies and standards are in place
- Standard operating procedures
  - Assay techniques and processes standardised
- Validated methods
- Appropriate quality control
- Staff requirements – training, competency
- Instrument and consumable Quality control
- Laboratory maintenance
- Appropriate facilities

Contamination

- **Amplicon Aerosols**
  - Single most NB source of PCR product contamination associated with post PCR analysis
  - Amplicons cannot be seen, felt, or detected before the contamination happens
- **Target Template Contaminants**
  - Where target template itself is the source of contamination – sample prep area and extraction areas.
  - Aerosols generated during specimen prep
  - Not following GLP during specimen preparation and extraction steps.
  - Repeated analysis of similar samples – diagnostic labs

PCR amplicon contamination

- control and removal of PCR amplicons form the basis of contamination control :
  - space and time separation of pre- and post-PCR activities
  - use of physical aids
  - use of ultraviolet (UV) light
  - use of aliquoted PCR reagents,
  - incorporation of numerous positive and negative or blank PCRs
  - chemical and biochemical reactions

Biochemical contamination prevention Uracil-DNA-glycosylase (UDG)

- Enzyme effective at destroying PCR amplicons during pre-PCR step,
- dTTP is substituted with dUTP, and UDG is included in the reaction mix.
- In the final product, there is now dU instead of dT in the DNA sequence→exposed to UDG enzyme.
- If UDG comes across any U-containing DNA strands, the U's are cleaved, leaving the strand with gaps→basic strands fall apart and cannot be amplified.

Uracil-DNA-glycosylase

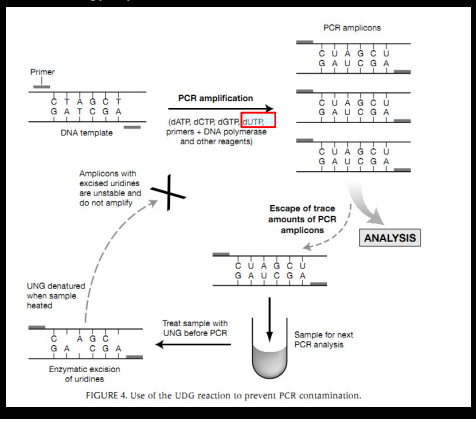


FIGURE 4. Use of the UDG reaction to prevent PCR contamination.

Molecular Lab Space and Design

- Limiting factor of PCR based technologies – Contamination – highly sensitive nature of PCR amplification
- Space and time separation of pre and post PCR activities
- Vital that correct workflow is followed – minimise contamination.
- Major separations –
  - Pre –amplification work – "Clean area"
  - Post PCR work – "Dirty area"

Environmental Considerations

- Air handling: air pressure
- UV radiation
- Dedicated lab coats
- Gloves available in all areas
- Non absorbent floors – cleaned regularly and in a controlled manner

LAB LAYOUT

CLEAN AREA
- Sample preparation
- Reagent Room
- Loading Room

ONE WAY TRAFFIC

DIRTY AREA
- Amplification Room
- Detection Room
- Blot Room

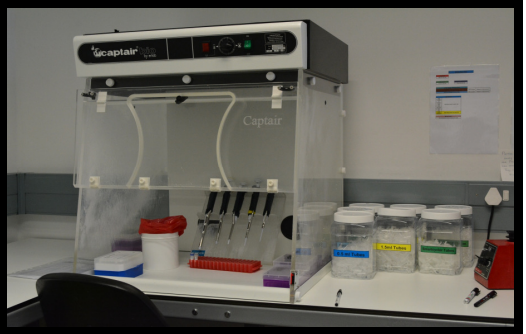
Clean area/rooms

- Specimen processing laboratory
  - Specimens received, processed and stored.
  - **Dedicated equipment normally found in Specimen processing laboratory**
    - Freezers and fridges for sample storage and extraction reagents storage.
    - Biohazard hoods for initial sample preparation (all specimens regarded as infectious)
    - Centifuges, microfuges
    - Dry heating blocks (with dedicated thermometers)
    - Dedicated pipettes (colour coded), filter tips, vortexes, timers
    - Vacuum manifold (manual extractions), semi – automated extraction platforms, fully automated extraction platforms
    - Storage space for tubes, pipette tips and other consumables.

No Template laboratory – reagent room

- PCR reagents stored, mastermix preparation for cDNA and amplification
- Positive air pressure
- **Free of amplicon at all times!!**
- Movement control / dedicated staff for each area on a rotational basis
  - Dedicated equipment normally found in a no template room
    - -20C Freezers, Fridge – reagent storage
    - Dedicated pipettes (Colour coded) – filter tips
    - Dedicated vortex
    - Dedicated microfuge
    - PCR workstations (with UV light)
    - Dedicated place to hang lab coats/ or disposable lab coats
    - All consumables necessary to perform work in area

No Template laboratory



Nucleic acid loading area

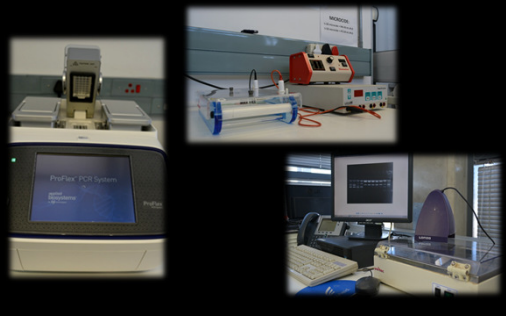
- Extracted nucleic acid added to master mixes
  - Dedicated equipment normally found in a loading area :
    - Freezer and Fridge (positive controls and nucleic acid storage)
    - PCR workstations
    - Dedicated minifuge
    - Dedicated pipettes (Colour coded) – filter tips
    - Dedicated vortexer
    - Thermal cycler (for cDNA synthesis only)
    - Gloves
    - Labcoats.

"Dirty areas"

- Depending on the molecular detection platforms used – this area can be divided into dedicated rooms /technology – depending on available space
  - Viral load platforms
  - Real-time PCR platforms
  - Thermal cyclers
  - Line probe assays(ELISA based detection) GT Blot
  - Sequencing platform
  - Gel electrophoresis
- Nothing from these areas should move back to the clean area!
- Gloves and lab coats to be removed when leaving this area!
- Dedicated pipettes, fridges, freezers, vortex, centrifuges.

Dirty area instruments

Gel electrophoresis



Sample preparation/extraction

- To Isolate nucleic acid of interest
  - Removes any potential inhibitors
  - Concentrates the nucleic acid
  - Increases the ability to detect very low concentrations of target nucleic acid.
- Pre-extraction sample preparation:
  - Problem area for developing fully automated systems – due to sample source variability.

Sample types

- Whole blood
- Serum
- Plasma
- Urine
- Stool
- Sputum
- Swabs – nasal, cervical, rectal
- Fluids – eye, amniotic,
- CSF
- Tissue
- BAL

Nucleic acid extraction

- Pre extraction steps: liquefaction, centrifugation, external lysis (lysis buffer, proteinase K, boiling).
- Manual extraction – vacuum/spin - column based
- Semi – automated – Nuclisens Easymag
- Automated extraction
  - More consistent results
  - Eliminates operator variability – manual methods
  - Reduces hands on time
  - Reduces transcription errors – labelling multiple tubes
- Increase in productivity
- Higher throughput

Extraction area

MagNApure 96

Common types of PCR used

- Conventional PCR
- Real-time PCR
  - Different fluorescent detection chemistries
    - SYBR Green I – intercalating dye
    - Dual-labeled fluorogenic oligonucleotide probes (TaqMan)
    - Flourescence resonance energy transfer (FRET) probes
    - Molecular Beacon probes
- Multiplex Real-time PCR
- Reverse – transcription (RT) PCR (Real-time)

Controls used in PCR runs

- Positive control
  - Contains target of interest and is known to work
  - NB for qualitative assays – identifies the amplification efficiency of the assay
- Negative control
  - Extraction negative control -Water/PBS specimen that is extracted and loaded on PCR.
  - Also controls for pipetting errors – during loading of speimens as loading order is always – specimens, pos control, neg control, blank.
- No template control (Blank)
  - PCR reaction without presence of starting material
  - Uses nuclease free water
  - Indicates if PCR reagents are contaminated
  - If positive – investigate , clean all pipettes, work areas and replace reagents – results cannot be used
- Internal control
  - Monitor nucleic acid isolation procedure
  - Indicates possible inhibition of PCR reaction.
  - Control is run in same tube as sample

Platforms used

- Conventional PCR
  - Thermal cycler, gel electrophoresis equipment, gel documentation system including UV transilluminator
- Real-time PCR/Multiplex PCR/RT-PCR
  - Roche lightcycler,
  - Rotorgene
  - Smartcycler
  - LC 480
  - Biorad CFX 96
  - Roche Cobas Ampliprep/Taqman
  - Abbot M2000 SP/RT

Cepheid-SmartCycler®

Random access instrument

Fluorophores
- FAM, Cy3/TET, Texas Red /ROX & Cy5

Detection
- Intercalating dyes (SYBR Green)
- TaqMan
- Molecular Beacons

Roche Cobas Ampliprep/Taqman

Roche Lightcycler

Using real-time PCR

- No opening of tubes = no amplicons released
- Drastically reduced the risk of amplicon contamination
- However: even these systems can have potential contamination issues:
  - Lightcycler glass capillaries can brake
  - Post Real-time containers not disposed of properly – spills!

Test Menu – Infectious disease Molecular Diagnostic Laboratory

- Virology
  - Quantitative assays
    - HIV, HBV, HCV, CMV, - automated platform
    - EBV – Real-time PCR
  - Qualitative assays – Real-time PCR
    - Adenovirus, CMV, EBV, EV, Influenza, HBV, HCV, HHV6, HPV, HSV, Mumps virus, Parvo, VZV, RSV.
    - JC/BK
  - Multiplex assays
    - Viral meningitis – HSV-1/2, EV, Mumps, VZV, Parechovirus
    - Respiratory Virus multiplex (16)
      Adeno, Inf A, B, RSV A, B, parainfluenza 1-4, HRV, EV, , MPV, HBoV, Corona (229E, NL63, OC43)

Gastro viruses multiplex – evaluation –Adenovirus, Astro, Rotavirus, Noro GI, GII, Sapo

Bacteria, parasites, fungi

- *Bordetella pertussis/parapertussis*
- *Brucella*
- *Clostridium difficile*
- *Chlamydophila pneumonia*
- *Clamydiae psittaci*
- *Legionella pneumophilae*
- *Mycoplasma pneumonia*
- *Toxoplasma gondii*
- *EPEC/EHEC*
- *Pneumocystis jiroveci*
- *Mycobacterium tuberculosis*
- *Malaria – species identification*
- *Aspergillus spp*
- *Ricketsia*

Real –time Multiplex: bacterial

- Respiratory bacteria – 5 plex
  - *Bordetella pertussis/parapertussis, Legionella pneumophila, Chlamydophila pneumoniae, Mycoplasma pneumoniae*
- STD – 7 plex
  - UU, UP, MG , MH , NG ,CT ,TV , IC.
- Carbapenemase – 6 plex
  - NDM-1, KPC, OXA,VIM, GES, IMP
  - IC

Genotyping/ Sequencing

- Hain TB drug resistance assay
  - 1st line and 2nd line drug resistance.
- Mycobacterium spp. identification (CM/AS)
- Sequencing:
  - HIV-1drug resistance testing
  - Panfungal
  - Panbacterial
  - HCV genotyping

Recent developments

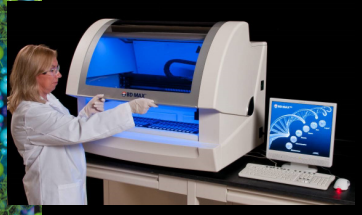
- Significant advances – microfluidics, micro-electronics and microfabrication
- Development of simplified molecular systems – possibility of sample to result automation
- Facilitating implementation in labs that lack capacity or expertise to perform molecular testing – potentially POC?
  - Cepheid- GeneXpert
  - Becton Dickenson BD Max
  - Idaho Technologies - film array

Cepheid-GeneXpert®

- Self-contained cartridges
- Fully integrated sample prep, amplification and detection
- 4 color detection
- TB, C.difficile
- POCT

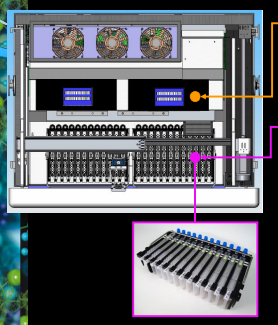
www.cepheid.com

BD Max



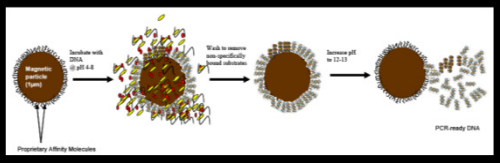
For BD internal Use

BD MAX Layout



- Two Readers
  - Independent thermal control
  - 24 lane PCR cartridge
  - 5 Color Detection
    475/520,530/565,585/630,630/665,680/715
- Two Sample Racks
  - 12 samples per rack
  - Mix compatible assays in the same rack
- Embedded Control System
  - Test and Worklist Set Up
  - Data Analysis and Reports
  - Export data

DNA Extraction



- ✓ Very Fast, Single Tube Process
- ✓ Extremely Effective Clean-Up For PCR
- ✓ Wide Variety of Sample Matrices

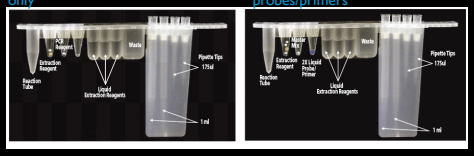
Creating a Test Protocol: DNA PCR Formats

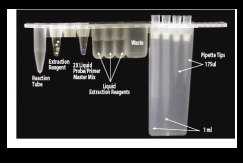
Type I (Closed):

Lyophilized master mix, IVD-assays only

Open:

Lyophilized master mix, liquid probes/primers

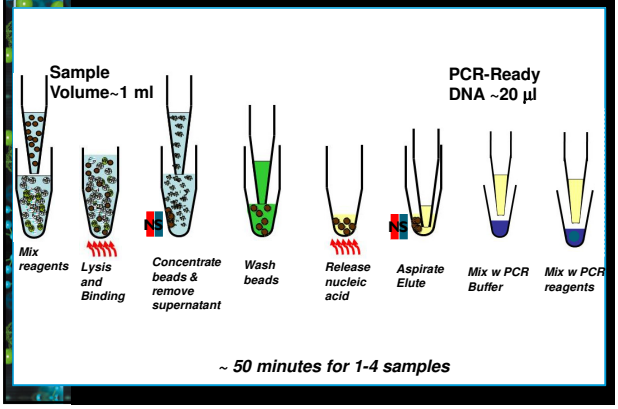




Research:

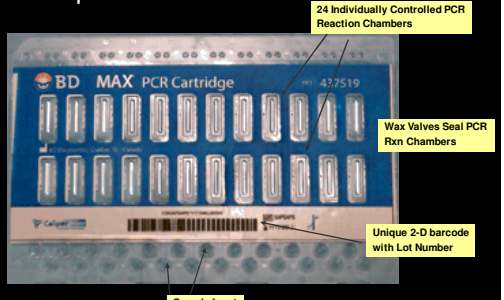
Liquid master mix, liquid probes/primers

Automated Extraction Process



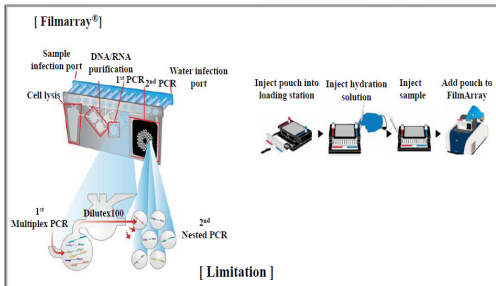
Microfluidic PCR Cartridge

- For amplification and detection



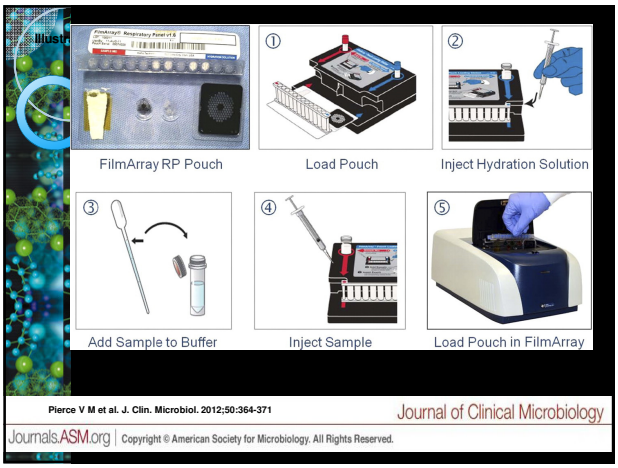
Technology for RV MoDx

FilmArray Respiratory Panel by FilmArray®



[Limitation]

1. No quantitative results
2. One analyte per operation => No high-throughput
3. Limited instrument



Film array system

- Fully integrated, closed system, minimizes contamination concerns – provides complete specimen to result automation
- Rapid, easy to use, multiplex format.
- 3-5 minutes hands on – 1hr to result
- Drawback – low throughput
- Failed runs rate 4.2%

Thank you for your attention

- (on a Saturday morning!)
- Questions?

Reference

- Raquel V. Viana and Carole L. Wallis (2011). Good Clinical Laboratory Practice (GCLP) for Molecular Based Tests used in Diagnostic laboratories., Wide Spectra of Quality control, Dr Isin Akyar (Ed.), ISBN: 978-953-307-683-6, InTech – http://www.intechopen.com/books/wide-spectra-of-quality- control/good-clinical-practice-gclp-for-molecular-based-tests-used-in-diagnostic-laboratories.