Molecular Laboratory Standards and Sources of Error

Reza Shahsiah, MD, FASCP

Tehran University of Medical Sciences

Challenges

- Nucleic acid testing for infectious diseases, human genetics, hematology, and oncology is the fastest growing field in laboratory medicine.
- Molecular methods are currently complex and involve multiple steps
- Interpretation of the results requires with clinical information

Pre-Implementation Considerations

- Clinical utility and test performance requirements
- Analytical and Clinical validity
- Personnel requirement,
- Facility and equipment
- Pricing, cost, Reimbursement

Personnel

Facility and Equipment

- Each method has different requirements
- If not addressed serious consequences
- *Facilities*
- For example, target amplification (PCR) is very sensitive to backflow
- – Incorrect patient diagnosis and patient management
- – Lack of confidence and test credibility
- – Negative impact on finances
- – Extensive laboratory clean up; laboratory closure; and retraining of personnel
- Unique Equipment for Molecular Assays
- – Micropipettes, thermal cyclers, real-time PCR units, imaging systems, hybridization ovens, capillary electrophoresis systems, microarray instruments
- – Cold storage of reagents (manual defrost refrigerators)
- • Separate storage for pre- and post-PCR

Analytical And Clinical Validity

- Analytical validation and performance must be determined before reporting patient test results
- The complexity of the validation process and parameters are related to the assay type: FDA-cleared or approved assays and CE-IVD-labeled assays need to be verified.
- Laboratory developed tests must have verification and validation on all aspects of the assay including reagent components, instrument, and software.

Reference and Control Materials

- Not all QC materials are reference standards
  - Test development and validation
  - Development of quality control material
  - Proficiency testing
  - Calibration
  - Internal vs external controls

QA Definition

Quality assurance encompasses all procedures and activities directed toward ensuring that a specified quality of product is achieved and maintained. Quality assurance systems and must address all phases as follows:

1. the preanalytical phase;
2. the analytical phase; and
3. the postanalytical phase

Activities

- Sample collection and receipt
- Nucleic acid extraction
- Nucleic acid quality and quantity assessment
- Analytical Phase Method
- Analysis and Release of results
- Equipment maintenance

Sample Collection

- Types and Volume of sample
- Timing for sample collection (ie, infectious disease)
- Transport stability (eg, RNA is less stable than DNA and Storage
- Special specimen acceptance criteria

Nucleic Acid Extraction

- Inhibitory substances (i.e Heparin)
- Fixatives used (i.e Formalin)
- Characteristics of nucleic acids: RNA vs. DNA; virus vs. genomic
- Characteristics of the sample: Stool vs. whole blood
- Quality and quantity: Nano-drop vs internal Control

Analytical Phase Methodology

- Each method has its own strengths, and caveats:
  - Target amplification methods are prone to post-PCR contamination
  - Sanger sequencing has a low LoD
  - NextGen Sequencing has a very complex workflow

Reagents

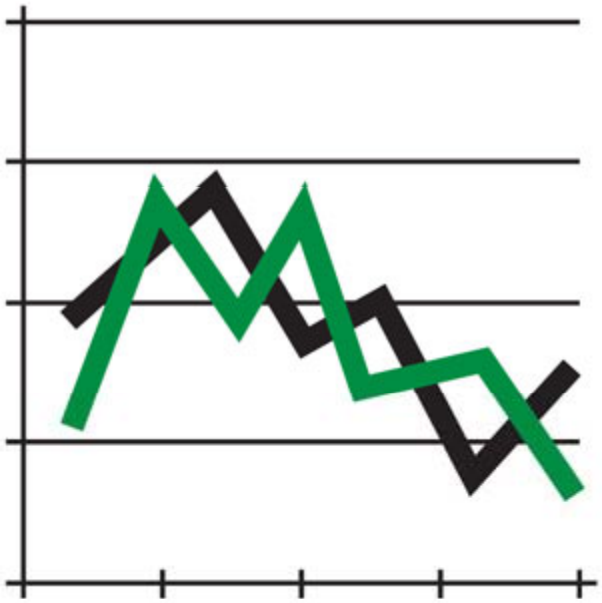
- Labeling Reagents:
  - Content, quantity, concentration
  - Lot#
  - Date or number of times used
- Storage requirements (temperature etc.)
- Expiration date
  - Date o fuse/disposal
- Critical reagents (enzymes, probes, digestion and electrophoresis buffers) may need QC checks as appropriate

Controls

- Internal Control to detect failure of DNA extraction or PCR amplification
  - Reagent or equipment issues
  - Integrity of DNA sample
  - Presence of inhibitory substance
- External Control
  - Positive control
  - Negative control (normal, wild type)
  - No template control (extraction blank)

Statistical Quality Control

In fact, only by running an independent control can a laboratory track trends and shifts in the manner that clinical chemists have traditionally done.



Proficiency Testing

- Assessment of the Competence in Testing
  - Required for all CLIA/CAP certified laboratories
  - Performed twice a year
- If specimens are not commercially available alternative proficiency testing program has to be established (specimen exchange etc.)

Reports

- Include indication for testing
- Have an interpretive category for the test result
- Specific methodology used
- Test limitations
- Sensitivity
- Relevant literature
- Recommendations for further testing, if needed
- ASR statement, if applicable

Traceability and Tracking

- Use two patient-identifiers at every step of the procedure
- Develop worksheets and
- Document every step

Pre-Analytical Errors

- Pre-analytical errors can occur at the time of patient assessment, test order entry, request completion, patient identification, specimen collection, specimen transport, or specimen receipt in the laboratory.

Error Prevention

- Developing clear written procedures.
- Enhancing health care professional training.
- Automating functions, both for support operations and for executive operations.
- Monitoring quality indicators.
- Improving communication among health care professionals and fostering interdepartmental cooperation.

Contamination

- Sensitivity of PCR techniques makes them vulnerable to contamination
- Sources of Contamination
  - Cross contamination between specimens
  - Amplification product contamination
- Routes of Contamination
  - Laboratory surfaces
    Ventilation ducts
- Reagents/supplies
  Hair, skin, saliva, and clothes of lab personnel

Laboratory design

Laboratory design is a key element of QA for molecular assay development and for routine testing inclinical laboratories. Ideally, for amplification-based assays, **three physically separate areas** should be available for reagent preparation (cleanroom), sample preparation and amplification, and product detection (postamplification). **Unidirectional workflow** must be maintained for molecular amplification procedures. The **preamplification laboratories should be under positive air pressure**

Facility and Equipment

- Each area has separate sets of equipment and supplies
  - Refrigerator/freezer (manual defrost)
  - Pipettes, filtered tips, tubes, and racks
  - Centrifuge, timers, vortex
  - Lab coat (color-coded), disposable gloves, safety glasses, and other PPE
  - Cleaning supplies
  - Office supplies
  - Ventilation system
- Dead air box with UV light – serves as a clean bench area

Sample Preparation

Even with closed systems, **sample preparation should minimally be performed in a class II biological safety cabinet** or maintained at a positive pressure to other areas.

Topical Inactivation Methods

- A useful and effective method is a 10% (v/v) dilution of sodium hypochlorite
- Ultraviolet lights placed over working surfaces may also be effective in controlling contamination with small amounts of nucleic acid (below 100 pg).

Contamination Control

- Maintain separate equipment and supplies for assay setup in the clean area from that used for handling specimens and extracting DNA.
- ❑ Always use aerosol-guarded (filter) pipettor tips to prevent contamination of pipettor barrels by aerosols; never reuse tips.

Contamination Control

- Change gloves between each step, or more often as needed, and when entering or reentering separate areas.
- Change laboratory coats when moving between areas.

Contamination Control

- Decontaminate surfaces daily with 10 to 20% bleach, followed by ethanol or clean distilled water, or as recommended by equipment manufacturers.
- Control airflow within and across work areas.

Contamination Control

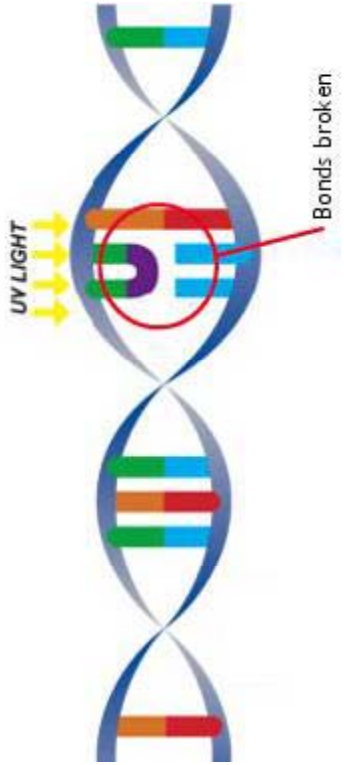
- Consolidate all handling of postamplification products and used materials in a defined area.
- Quick-spin tubes to force any liquid down from the sides, before removing caps. Tubes should be uncapped carefully to prevent aerosols and recapped as soon as transfers are completed.

Contamination Control

- Keep reagent tubes capped unless in use.
- Dispense negative controls last so that they reflect the cumulative effects of manipulations.

UV

Ultraviolet treatment of DNA induces crosslinking of the two strands of DNA by forming thymidine dimers . This crosslinked DNA can no longer serve as an effective template .One disadvantage of ultraviolet treatment is that it is most effective in sequences over 700 nucleotides in length .

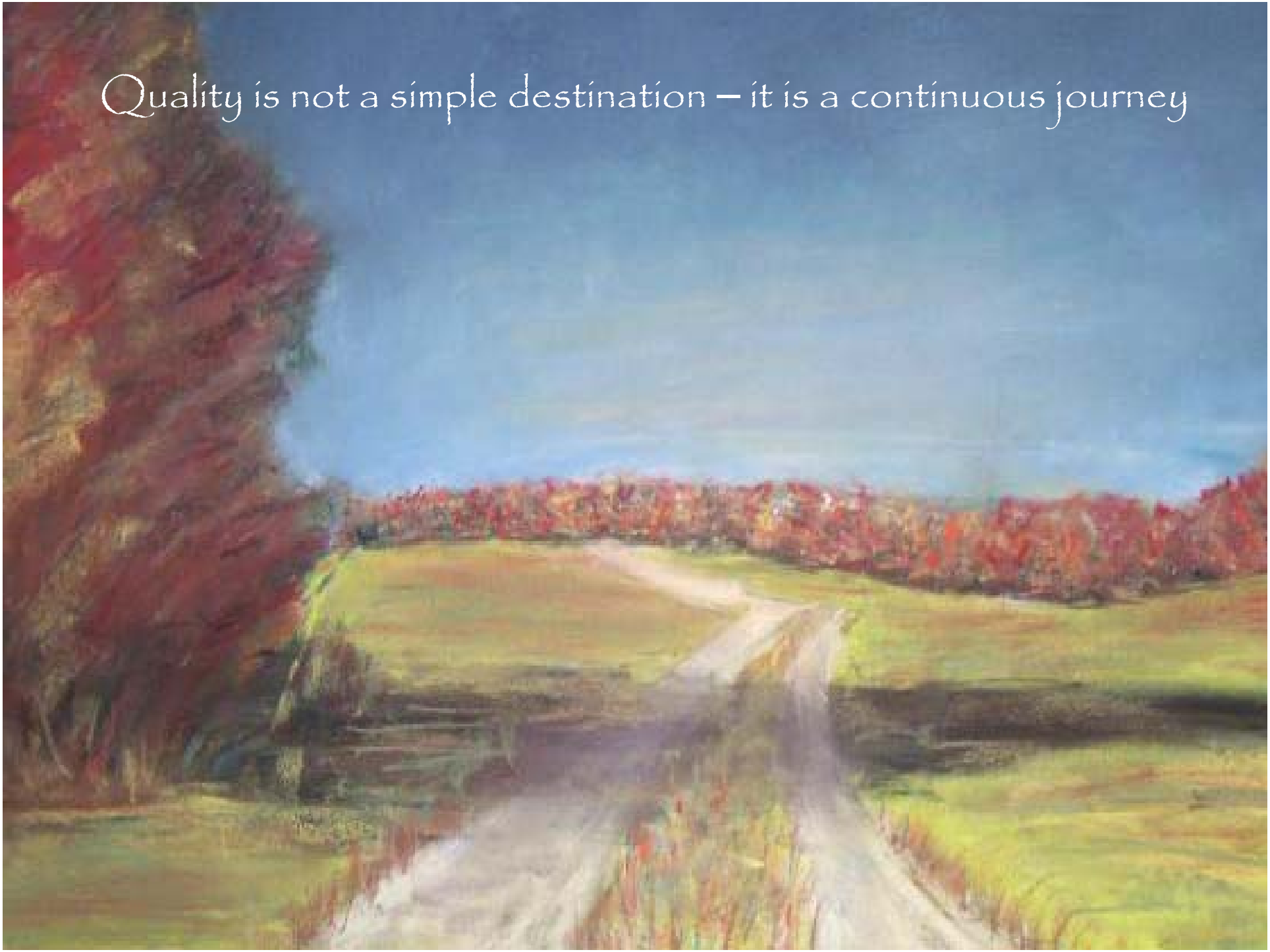


Photochemical Inactivation

Isopsoralen compounds are added to the reaction mixture prior to amplification. Following polymerase chain reaction (PCR) but before the reaction tube is opened, the vessel is exposed to ultraviolet light (300–400 nm), which activates the isopsoralens to form adductors between the pyrimidines on the amplicons. These adductors stop *Taq* polymerase from processing along the amplicons and thus prevent subsequent reamplification of any of these contaminating amplicons

Enzymatic Inactivation

UNG removes uracil residues from the amplicon, still leaving an intact phosphodiester backbone on the amplicon. During the first denaturation step of the amplification procedure, the phosphodiester bonds break at the sites where the uracil residues were located. The fragmented amplicon is no longer able to act as a template. Use of deoxyuridine triphosphate and UNG have been shown to be effective in controlling contamination if amplification of the uracil-containing amplicon does not exceed 106–107 copies per reaction.


Quality is not a simple destination – it is a continuous journey