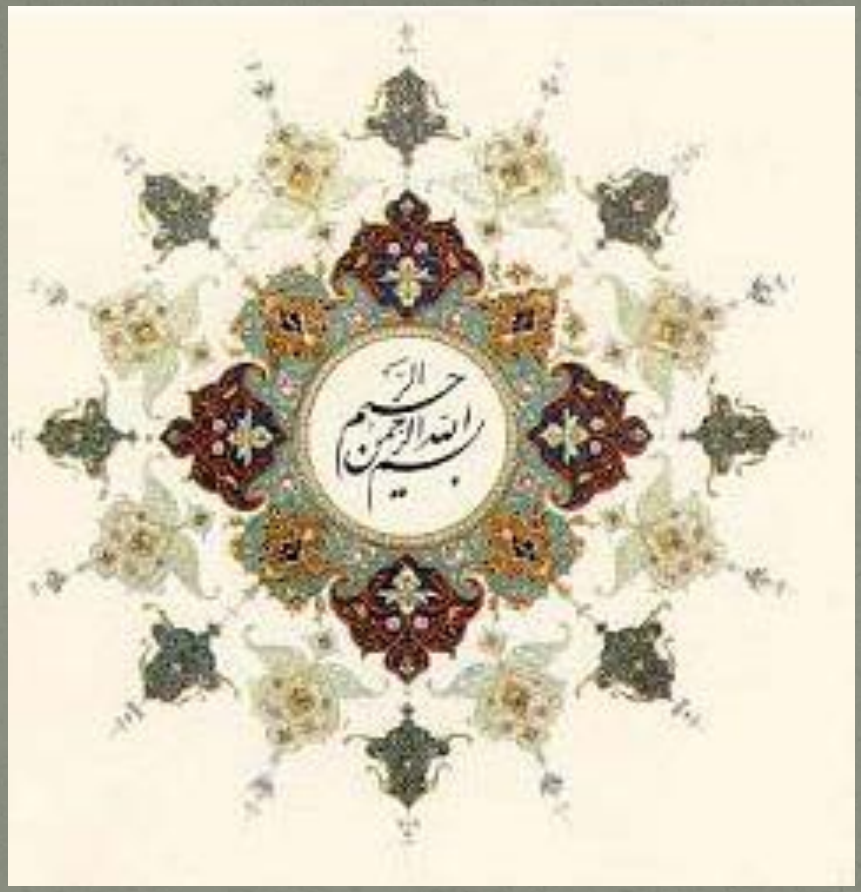
بسم الله الرحمن الرحیم

CHECKLIST FOR OPTIMIZATION AND VALIDATION OF MOLECULAR ASSAYS

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Choice of Method

- Commercial assay or in-house assay
- Choice of target gene
- Choice of detection method
- Choice of oligonucleotides

Choice of Method

- **In-house assay:**

✓ which need adequate optimization and validation before they are introduced in routine diagnostics

- **Commercial assay:**

✓ FDA or IVD-CE labeled assays, the manufacturer is responsible for the performance as stated in the kit insert. Nevertheless, the user should verify that the indicated performance characteristics are achieved in the local laboratory

Verification Guidelines

ASR: Analyte Specific Reagent

Choice of target gene

- For viruses and bacteria, a specific and conserved nucleic acid target sequence is selected

- For reverse transcription (RT) such as hematological assays, primers and probes should span an exon–exon splice junction, This prevents coamplification of genomic DNA

Choice of detection method

- The detection method used at present in real-time PCR is monitoring of fluorescence

- Nonspecific labels (intercalating dyes) and sequence-specific probes are available as reporters

Choice of oligonucleotides

- The use of optimal primer and probe sequences is one of the critical steps for a successful PCR.

- ***Tm of primers: 58–60°C***
- ***GC content of oligonucleotides: 30–70%***
- ***Not more than two C or G in last five positions at 3' end of primer***
- ***Length of amplicon: max 400 bp***
- ***No more than four constitutive guanines***
- ***Avoid primer–dimer***
- ***Length of primer: 18–24 base pairs***
- ***Tm of probe: 68–70°C***
- ***More C than G in probe***

Choice of sample material and sample processing

- A sample material-specific validation approach is absolutely necessary because of possible matrix-induced effects
- The performance of a diagnostic PCR may be limited by the presence of inhibitor substances within individual samples
- Correct sample processing should remove PCR inhibitors, concentrate the target nucleic acids, and turn a heterogeneous biological sample into a homogeneous PCR-compatible sample

Quantification strategies

- **Standard curve method:**

✓ Requires the construction of a standard curve, plotting the Ct values against the logarithm of the initial copy numbers of standards with known concentration

- **Comparative method:**

✓ Determines the changes of steady-state transcription of a gene

- **Validation:**

✓ is the process of establishing documented evidence that provides a high degree of assurance that a product, service, or system accomplishes its intended requirements

- **Verification:**

✓ is a process that is used to evaluate whether or not a product, test, or system complies with a regulation or conditions imposed at the start of a development phase

Validation

- **Verification of design of oligonucleotides:**

✓ The specificity of the amplicon is verified by using the BLAST algorithm, expectation value ≤0.01

- **Verification of amplification:**

✓ The absence of primer–dimer formation should be checked by melt-curve analysis

✓ The length of the amplicon, analyzed by gel electrophoresis

✓ The amplification product must be analyzed by Sequencing+blast of amplicon

Optimization of reaction conditions

- **Optimization of primers and probe concentration**

✓ Reduce primer–dimer formation and increase the efficiency and specificity of the amplification process.

- **Optimization of annealing temperature**

✓ Software programs do not account for the stabilizing effect of the Taq polymerase, making optimization of the annealing temperature necessary.

- **Optimization of sample input**

✓ The DNA/cDNA input must be optimized to ensure a maximal sensitivity with minimal inhibition.

PCR characteristics

- **Slope m:** Ct=log conc. × m + y-intercept
  (criterium: $-3.6 \leq m \leq -3.1$)

- **Efficiency E:** $E=10^{-1/slope}-1$ (criterium: $0.9<E<1.1$)

- **Coefficient of correlation r^2:**
  (criterium: $0.99 \leq r^2 \leq 0.999$)

Analytical verification

- Precision
- Linearity, measuring range
- Trueness
- Limit of detection (≥95%)/limit of quantification
- Analytical specificity

Clinical verification

- Clinical question (Critically Appraised Topic ,CAT)
- Clinical performance
- Correlation to disease or disorder

✓ – Negative predictive value

✓ – Positive predictive value

- Comparison to current methods/standards

Internal quality control

- Amplification and inhibition control
- Negative control
- Statistical follow-up of a positive control

Amplification and inhibition control

- Amplification of an internal control (IC) must be included in every assay to exclude false negative results due to interference of inhibitors and to ensure the performance of the nucleic acid extraction procedure

✓ ***Human gene as IC: cell-rich specimens***

✓ ***Synthetic IC: cell-free specimens***

Negative control

- Analysis of a negative control, simultaneously with the specimens, enables detection of possible contamination during the extraction or the amplification

- Also, the specificity of the assay can be demonstrated

Statistical follow-up of a positive control

- **Reference material is necessary**, the concentration of the control should be near the limit of detection of the assay but high enough to obtain reliable results

- **For quantitative assays**, at least two concentrations of reference material should be tested

Proficiency testing

- External quality assessment is necessary for each assay that is performed, if available.

- If an external proficiency program survey is not available, alternative testing can include:

✓ Blind sample testing

✓ Exchange of samples with other laboratories

✓ Medical chart review, should be conducted yearly twice

CONCLUSION

- We hereby propose practical guidelines for the optimization and validation of commercial and in-house developed Molecular assays

- The proposed checklist is a crucial step in harmonization of different methodologies

Thank You For Your Attention