



## Association for Molecular Pathology

Promoting Clinical Practice, Basic Research, and Education in Molecular Pathology

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### **Certified Standards are Critical to Continued Innovation in Healthcare and the Realization of Personalized Medicine**

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#### **Background**

The modern healthcare system offers great potential for personalized and effective medical care. However, the recognition and implementation of advances in medical research may be hindered by a lack of certified reference materials. Molecular genetic assays provide the cutting edge for many individualized therapies in oncology, transplantation, infectious disease and genetics, but the production of certified reference materials has fallen far behind the technical capabilities of these assays. Reference materials are important to ensuring the necessary sensitivity, specificity and level of reproducibility of intra- and inter-laboratory test results. The best approach to achieve consistent and comparable quantitative data amongst laboratories is by the use of internationally established reference reagents.<sup>1</sup>

#### *Example 1: Targeted therapeutics and tumor markers*

Challenges in the delivery of molecularly targeted cancer therapy and the need for reference standards can be illustrated with selected examples from the treatment of leukemia and carcinoma. Historically, patients with chronic myeloid leukemia (CML) had few treatment options other than transplantation, but a recently developed novel class of medicines has revolutionized the treatment of CML. Tyrosine kinase inhibitors (TKI) are specifically targeted against the oncogenic BCR-ABL fusion protein that results from the pathogenic translocation in CML, resulting in effective control of the tumor cells with relatively few side effects. The standard of care for monitoring the effectiveness of CML therapy is the quantitative molecular assessment of the level of the BCR-ABL fusion gene in the patient's blood. Rising levels of the fusion gene indicate the risk of relapse and the need to alter therapy. Accurate assessment of the level of BCR-ABL is essential for both the individual patient and to allow medical centers to accurately compare results from clinical trials for improvement of leukemia therapy. Patients with rising BCR-ABL levels will also be evaluated by sequencing for novel resistance mutations in BCR-ABL and therapy will be redirected with a different TKI (or altered dose) that retains efficacy against the novel mutation.

These advances have obviated the need for transplant in most CML patients, but the lack of standardized reagents has limited the reproducibility of these assays within and between laboratories. Thus, once a patient's blood has been analyzed by one laboratory, all subsequent testing needs to be done at the same site, thereby limiting the patient's healthcare choices. Therefore, quantitative standards for monitoring BCR-ABL are urgently needed. Furthermore, the model provided by CML may become the standard for other genes with molecularly targetable mutations or mutations suitable for minimal residual disease monitoring; e.g. PML-RARA and variants, FLT3, cKIT, PDGFRA, PDGFRB, NPM1, ETO-AML1, JAK2, MLL-mutation variants, etc.

*Example 2: Companion diagnostic tests*

Molecularly targeted therapies are frequently expensive and sometimes have significant side effects. Molecular pharmacogenomics assays (also called companion diagnostics) can be used in these cases to identify patients likely or unlikely to benefit from these therapies, providing a method for optimizing the cost-effective delivery of healthcare. This is exemplified by the recent recognition of the role of *KRAS* mutations in colorectal cancer to identify patients unlikely to benefit from monoclonal antibody therapy (Cetuximab/Erbitux) that inhibit the epidermal growth factor receptor (EGFR). Mutations in *KRAS* preclude response to this therapy. However, the sensitivity and specificity of different molecular assays for identification of *KRAS* mutations varies with technique. Furthermore, some activating mutations of *KRAS* have been identified for which the degree of resistance to anti-EGFR therapy is unknown. Standardized reagents are urgently needed to allow comparative analysis between clinical protocols. Mutations in the *EGFR* gene itself also appear to predict responsiveness to EGFR small molecule tyrosine kinase inhibitors (TKIs) in non-small cell lung cancer (NSCLC). However, *EGFR* mutations are considerably more varied than *KRAS* mutations, demonstrating the need not only for standardized reagents but also for an up-to-date *EGFR* somatic mutation database that can predict TKI response in NSCLC for individual mutations.

*Example 3: Transplant follow-up care and quantitative standards*

Standardized molecular reagents are also urgently needed in the transplant setting. At the end of 2006, over 170,000 people in the U.S. were living with a functioning solid organ transplant; 27,578 solid organ transplants were performed in 2007.<sup>2</sup>

From its founding in 1986 through 2004, the National Marrow Donor Program® coordinated more than 20,000 bone marrow and peripheral blood stem cell transplants.<sup>3</sup>

All patients who have undergone transplants are given immune-suppressive drug therapy and are as a result more susceptible to viral and fungal diseases. Viral diseases can result via transmission from the donor tissue, exposure to the environment or more typically, reactivation of the patient's own latent viruses, held for years within their own body. Several common viruses represent recognized and severe complications of organ and bone marrow transplantation, which adds excessive costs to US healthcare systems. Quantitative testing for viruses (viral load testing, e.g. for CMV, EBV, and BK viruses) is considered standard of care for perpetual monitoring of transplant patients. However, laboratory tests show marked variability among commonly used methods because there are no established quantitative virus standards. Because of the variability among laboratory tests, repeat testing is often required when a patient switches health insurance or travels to a different hospital or city. Therefore, quantitative virus standards are urgently needed by the clinical laboratory community in order to provide accurate reproducible and comparable results to physicians and to limit errors and repeat testing, which add to overall healthcare costs for this already costly group of patients.

Adenovirus infection is a life threatening condition in immunosuppressed patients, and transplant patients in whom bloodstream infections, hemorrhagic kidney disease, and diarrhea can be deadly. Quantitative assays are important tool for directing and monitoring antiviral therapy in these patients. Adenovirus viral load testing will have much the same benefit as that of CMV and other viral loads in these patients.

*Example 4: Reference gene sequence database*

Currently, clinical sequencing methods rely on the use of public sequence databases for sequence comparisons, or labs must pay high proprietary fees to commercial companies that annotate the sequences. Genetic sequence banks such as GenBank are unacceptable for use in clinical laboratories because of the open platform for those who enter sequences and the limited sequence verification for GenBank submission. Clinical laboratories performing gene analyses need a “certified” reference sequence that is locked and annotated.

Clinical laboratories rely on accurate genotypic bacterial identification based on the 16S rRNA gene for many fastidious microbes and fungi. The use of the 16S rRNA gene for identification of bacteria and fungi provides a faster method to identify slow growing organisms. Traditional methods may take up to 3 weeks to identify the microbe and delays in adequate treatment can occur, causing mortality and increasing hospital costs and in some cases, like tuberculosis, a risk to the public health. Two databases services, freely available on the Internet, offer an improved scenario, with some level of verification: 1) The Ribosomal Differentiation of Medical Microorganisms (RIDOM), and 2) the Ribosomal Database project (RDP) from the University of Michigan. These databases offer improvements in secondary-structure based alignment that provides better support for short partial sequences and improves handling of certain sequencing artifacts. However, limitations exist with these databases including, but not limited to, limited species representation and research use only disclaimers.

While improved over GenBank, experience in the clinical laboratory with analysis of the 16S rRNA gene of patient strains has shown that clear-cut results are not the rule as the existing databases are not always well annotated or regularly updated for taxonomy with the speed that medical laboratories require. This may be due to the lack of coordination with these services and actual medical/clinical laboratories, as these sites still mainly rely on researchers to submit microbial sequences. Commercial databases, such as MicroSeq (Applied Biosciences), and databases from reference laboratories such as Mayo Clinic and ARUP Laboratories offer better association with clinical laboratories, yet still rely on the subset of microbes submitted to them for identification to populate their databases.

As many clinical, research, and environmental laboratories currently use 16S-based identification of bacteria, including mycobacteria, a widely available quality-controlled database that interfaces freely and seeks to populate it with medically identified microbes from across the globe is long overdue. It is essential to accurately identify species or detect true sequence variations leading to the discovery of new species, with data validation protocols akin to that of 21 CFR Part 11 compliance. Ideally, such a database would provide ribosome related data and services to the clinical community, including online data analysis and aligned and annotated Bacterial and Archaeal small-subunit 16S rRNA sequences, as well as fungal rRNA sequences, and genetic sequences related to antimicrobial resistance. In terms of "personalized medicine" this resource would be valuable as sequence analysis of resistance mutations will be integral to initiatives such as personalized anti-tuberculosis (TB) therapy so that clinical laboratories could quickly identify drug resistant TB (MDR and XDR TB), a priority identified by the NIH.

The need for certified reference sequences extends to human genes, for both inherited diseases and acquired disorders (cancers). For example, the *RET* proto-oncogene is associated with Multiple Endocrine Neoplasia Type 2 (MEN2). From GenBank, two isoforms are given as well as alternative assemblies. One reference sequence lists a known minor allele as the as wildtype allele. Reference sequences differ by using only the coding region or the genomic sequence. The genomic sequence may use the 5' untranslated region or begin with the first base

of the transcribed mRNA. All these differences could cause confusion between reports from different laboratories testing for the same disease, based on which reference sequence is used. A clinically certified reference sequence would be checked to determine that the most common sequence is listed as the reference, and document known benign SNPs. The reference sequence would ideally have the chromosome, locus and sequence numbering so that results from different laboratories will be consistent. Annotating positions of the SNPs could help in designing assays and choosing primers avoiding the SNPs to reduce the potential of allele drop out and therefore false negative or positive results. A list of known benign SNPs can also help in interpretation when these variants are detected.

### **Priorities: Standards are urgently needed**

While in the end, we hope to have standardized reference materials for all diagnostics targets and certified reference databases for all clinically relevant gene sequences, some are more urgently needed than others.

1. Immediate
  - a. Cytomegalovirus (CMV), quantitative assay standard, a recognized complication of organ transplantation
  - b. BCR/ABL Adelaide standard; BCR/ABL tests are used to diagnose patients with a specific leukemia and to monitor their response to treatment
  - c. KRAS mutation standards; KRAS mutation analysis testing is used to select patients for a specific chemotherapeutic drug
  - d. EGFR mutation standards; EGFR mutation analysis testing is used to select patients for a specific chemotherapeutic drug
2. Medium term (one year) - all quantitative assay standards.
  - a. BK virus (BKV), a recognized complication of kidney transplantation
  - b. Epstein Barr Virus (EBV), a recognized complication of organ transplantation
3. Long term (1-3 years)
  - a. Adenovirus, quantitative assay standard; important for directing antiviral treatment in immunosuppressed patients
  - b. Enterovirus, qualitative assay standard
  - c. Hepatitis B virus (important for liver transplants and also in the general population), quantitative assay standard
  - d. Herpes simplex (HSV), types 1 and 2, qualitative assay standard, recognized complications of organ transplantation
  - e. HHV-6, HHV-7, and HHV-8, increasingly common complications of organ transplantation, which may add severity to the more common CMV infections
  - f. HTLV 1 and 2, qualitative assay standard, important for transfusion services
  - g. Human metapneumovirus (HMPV), qualitative assay standard
  - h. Influenza virus, qualitative assay standard
  - i. JC virus, quantitative assay standard, closely related to BKV
  - j. Parainfluenza virus, qualitative assay standard
  - k. Parvovirus B19, quantitative assay standard
  - l. Respiratory syncytial virus (RSV), both qualitative and quantitative assay standards; quantitative assays are used as prognostic markers for patient care
  - m. Varicella zoster virus (VZV), recognized complication of organ transplantation

- n. Certified Gene Sequence Databases (CGSDs)
  - (1) Gene mutation sequence database, suitable for clinical test reference
  - (2) Infectious agent (bacteria, viruses) sequence database, suitable for clinical test reference
- o. Scientific advisory committee to identify and prioritize areas of needed references materials and to direct resources and the work of the CGSDs

### **AMP's Ongoing Efforts**

The goal of the AMP Clinical Practice Committee is to increase the speed with which the National Institute for Standards and Technology (NIST) can prepare quantitative standards, which is critical to the national and international laboratory community and their ability to deliver accurate test results. The deliverable would be purchasable standardized reference materials that would ideally be available for inter-laboratory comparison studies and purchase by commercial and clinical laboratory communities.

The CGSD requires a synergy of efforts from international standards organizations, references laboratories, commercial organizations, and laboratory faculty who are most knowledgeable in the development of molecular diagnostics. AMP stands ready to collaborate with NIST and do its part to hasten the process to achieve available certified reference materials for all clinical tests.

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<sup>1</sup> Robertson JS. "International standardization of gene amplification technology." *Biologicals* 26:111-3, 1998.

<sup>2</sup> Wolfe RA, *et al.* "Trends in organ donation and transplantation in the United States, 1998-2007." *American Journal of Transplantation* 2009;9(Part 2):869-878.

<sup>3</sup> U.S. Department of Health & Human Services Health Resources and Services Administration (HRSA). *2004 Biennial Report of the National Bone Marrow Donor Registry*. Available online at [http://www.marrow.org/ABOUT/Who\\_We\\_Are/Publications/2004\\_Biennial\\_Report/PDF/biennial\\_report\\_2004\\_1.pdf](http://www.marrow.org/ABOUT/Who_We_Are/Publications/2004_Biennial_Report/PDF/biennial_report_2004_1.pdf)