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IN-BRIEF

Forensic Technology Center of Excellence:

Applying Internal Validation Guidelines for Expanded STR Loci Kits

Moving Knowledge from Research to Impact

"The new expansion includes loci aimed to become more compatible with international databases, increase the power of discrimination, and reduce the opportunity for accidental matches as the CODIS database expands in size."

Hares, Douglas R.
Selection and implementation of expanded CODIS core loci in the United States. Forensic Science International: Genetics 17 (2015) 33-34.

Introduction

The familiar '13 Combined DNA Index System (CODIS) core loci' expanded to '20 CODIS core loci' as mandated by the FBI on January 1, 2017. These additional loci were selected based on feedback obtained from the CODIS Core Loci Working Group and consortium testing of new Short Tandem Repeat (STR) kits at 11 laboratories across the United States. Due to conformance with previous DNA profiles already stored in the CODIS database, the original 13 loci will be maintained as required. The new expansion includes loci aimed to become more compatible with international databases, increase the power of discrimination, and reduce the opportunity for accidental matches as the CODIS database expands in size[1].

Because of this FBI mandate, the participating CODIS laboratories will need to internally validate an expanded STR loci kit for implementation in 2017. Information can be extrapolated from the Scientific Working Group on DNA Analysis Methods (SWGDAM) basic recommended internal validation studies such as concordance, sensitivity levels, analysis thresholds, precision, accuracy (repeatability and reproducibility), optimal DNA input ranges, detection of stutter or other artifacts, and system contamination. In addition, other trends such as when to distinguish major and minor DNA profiles.

Expanded STR at-a-glance

- **Original 13 CODIS core:** CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX, and vWA
- **Additional CODIS core:** D1S1656, D2S441, D2S1338, D10S1248, D12S391, D19S433, and D22S1045
- Several 'expanded STR kits' have been approved by the National DNA Index System (NDIS) that include the recommended 20 CODIS core loci: AB GlobalFiler™Express, AB GlobalFiler™, Promega PowerPlex® Fusion, Promega PowerPlex® Fusion 6C, QIAGEN Investigator 24plex GO!, and QIAGEN Investigator 24plex QS[2].

This Forensic Technology Center of Excellence (FTCoE) validation in-brief serves to provide application of already existing guidance documents. Organizations like SWGDAM and the Organization of Scientific Area Committees (OSAC) provide the forensic community best practices and are continuously improving standards and recommendations as technology improves and changes. The recommended validation studies are foundational for laboratories to determine the effectiveness and reliability of any new system to develop standard operating procedures (SOPs).

Sample Selection and Size

Samples for internal validation studies can be created using positive DNA standard reference materials, commercially available through the National Institute of Standards and Technology (NIST) standard reference materials (SRMs), and/or samples previously characterized by the laboratory (i.e. 9947, 9948, 2800M, and 007). The genotypes and the quantity of the selected samples should be known to aid in setting up successful validation studies. Using samples with a high level of heterozygosity and differing allele calls will provide for better indicators of kit performance as more information can be inferred from the profiles. Negative amplification controls such as water or Tris EDTA (TE-4) should be included throughout studies to focus on the performance of STR kit reagents.

The 2004 SWGDAM Validation Guidelines for DNA Analysis Methods recommended testing a minimum of 50 samples. In later revisions, this guidance was removed in part to allow for the laboratories to decide how many samples are needed to demonstrate if procedures work as expected, and to support the development of SOPs in their own jurisdictions. However, statistically using a student's t test, approximately five replicate experiments are a good foundation as based on 95% confidence intervals, there are diminishing returns capturing variability of data with each successive experiment beyond five replicates[3].

SWGDAM Recommended Studies (2012)[4]

Known and nonprobative evidence samples or mock evidence samples

It is important to include samples where the profiles are already known and well characterized when validating a new kit in order to verify concordance and begin to understand profile quality indicators such as peak height balance and baseline noise when compared to previously generated profiles. These samples will also help ensure the new kit chemistry is what is being tested and that the sample(s) is not a variable when exploring the limits of the new kit. The samples in this study should represent typical samples seen in forensic casework such as inhibited, degraded, low quantity, single source and mixed samples; or in forensic databasing, known reference samples on typically submitted substrates.

Sensitivity and stochastic studies

Sensitivity studies are used to determine the upper and lower limits of a kit such that the laboratory understands the quality of the profile when too much or too little DNA is amplified and to determine the ideal target amount. The samples included in this study also provide information on limit of detection, limit of quantitation, heterozygote balance, peak height ratios, PCR stutter artifacts, stochastic variation, and signal-to-noise (S/N) ratio. When developing a sensitivity test plan, explore the lower limits of detection and ideal target range expressed through the published developmental validation and build a dilution series around those values. For example, when validating a kit with an ideal target concentration range of 0.5 ng, a dilution series of 4ng, 2ng, 1ng, 0.5ng, 0.25ng, 0.125ng, 0.063ng, 0.031ng, and 0ng amplified in triplicate could be ideal. The dilution series may need to change if evaluating different Polymerase Chain Reaction (PCR) cycle numbers, electrophoretic injection times, reaction volume or other variables that affect sensitivity. Databasing laboratories may need to test higher input samples, and laboratories performing touch DNA may need to test additional low input samples. By amplifying 0ng (i.e., an amplification blank) the resulting data can also be used for a trusted assessment on contamination.

The sensitivity dilution series are also the samples that will be applicable for determining Analytical (AT) and Stochastic Thresholds (ST). Although negatives and blanks can be used to calculate AT, it is recommended that samples containing DNA are used to calculate ATs as amplification noise may change with DNA input[5]. The effects of DNA input on noise becomes significant with higher inputs of DNA such as those experienced in a database setting. The S/N ratio should be analyzed to determine the point at which noise artifacts become distinguishable from real data[6]. Setting an analytical threshold that is too high will result in loss of data, whereas setting it too low can confuse PCR artifacts with real data, confounding the profile. Although it is less complicated to have one AT established for a kit, it is common for forensic laboratories to have a different AT for every dye channel as the noise levels can vary between dyes, and analysis software can now accommodate the setting of different ATs per dye.

The AT will help to determine the ST as the lowest expected peak height ratio connects these two values[6]. The ST is defined as a value whereby above the ST the homozygosity of an allelic peak can be considered homozygous and below the ST the homozygosity is questioned due to the possibility of allelic drop out[7]. The ST can be determined by examining heterozygous loci in samples and noting from the peak height data when drop out occurs through use of a logistic regression curve[6,7]. Similar to assigning an AT, if the ST is too high there could be loss of data, whereas setting it too low, there would be a risk of reporting a false homozygote. The ST has further implications in analysis of mixtures depending on the statistical method used such as combined probability of inclusion/exclusion or semi-continuous probabilistic genotyping. With these methods, a

locus that displays alleles below the ST should be excluded in the calculation[8], barring any assumptions. Using a ST may not be necessary with continuous probabilistic genotype methods but the statistical validation can address this.

Precision and accuracy

Precision refers to the closeness or mutual agreement among a set of measured values (i.e., base pair sizes), whereas accuracy refers to the closeness of a measured value to a known value (i.e., known genotype). Under this category of tests, repeatability is examined by having the same operator perform an amplification of a replicate sample and reproducibility is examined by having different operators perform an amplification of a replicate sample. The kit should demonstrate that its results are precise (i.e., base pair size, allele calls), accurate (i.e., concordant genotypes) and not variable between operators.

Precision can easily be evaluated by examining allelic ladders in replicate injections whereas the sensitivity and known/non-probative sample studies can be used to test accuracy if designed appropriately to account for testing accuracy, repeatability and reproducibility.

Mixture studies

In databasing laboratories, a condensed mixture study can be useful for training to recognize mixtures and possible contamination. In casework laboratories, mixture studies are critical for developing internal mixture guidelines based on the kits performance in the laboratory. Adequate mixture studies should be performed that can provide guidance to determine the number of contributors and understand mixture proportions and ratios to be able to distinguish major and minor profiles. The samples chosen for this study should be representative of what is typically encountered in the laboratory such as varying mixture ratios and contributors, touch samples, and post-coital samples. Using samples with a high level of heterozygosity and varying alleles will allow for more apparent patterns to be recognized. The laboratory should create mixture ratios for the number of contributors it will be interpreting. For example, if it will be common to encounter and interpret mixtures of three people; then 3-person mixture ratios should be included as part of the basic study to create more robust interpretation protocols. Creating mixture ratios with varying percentages of a minor contributor(s) can be important to recognize when a major contributor can be analyzed from a profile with confidence.

Contamination Assessment

The laboratory should determine whether the new kit being validated, in conjunction with its sample handling procedures, introduces exogenous DNA that could adversely affect the integrity of the results. Negative samples should be included in the validation process to be able to test reagents, consumables, operator handling, and the laboratory environment with a focus on the processes used in amplification. To evaluate the possibility of drop-in or amplification related reagent dye artifacts, negative controls such as water or TE-4 should be used.

Additional studies

Once the basic studies have been completed, the data should be evaluated to determine if the performed studies support SOP development. The laboratory will have an indication that enough samples have been completed when the kit is demonstrated to be robust (successful results obtained a high percentage of the time), reliable (results are accurate and expected), and reproducible (the same or similar results are produced each time) [6]. Additionally, the laboratory must be able to support elements of guiding an analyst through the laboratory procedure, analysis, and interpretation of results through the validation data.

During experimental design, exploring which laboratory factors or equipment may need to be altered during validation to account for possible variability introduced by ordinary adjustments should be considered.[9] Examples of ordinary laboratory alterations may include, the extraction method, the quantification method, pipettes, capillary lot numbers, thermocycler, and amplification kit lot.

Even after a procedure has been implemented, there may be circumstances which require revisiting the validation study and performing additional tests to modify a procedure. Additional tests may be warranted to evaluate reducing PCR cycle number in response to large stochastic variations, performing additional mixture studies to create well rounded interpretation procedures, or as a response to changes in 'best practices' that may require reevaluating current procedures.

Data Analysis

After validation design, running samples, and collecting data, laboratories must analyze the data in order to extract trends, noise levels, appropriate threshold settings, and other valuable conclusions. Microsoft® Excel® is a widespread and well-known program for compiling, sorting, and statistically analyzing data. Additional software is available that laboratories may consider

obtaining to assist with their data analysis. A free suite of software, R, has environments for data manipulation, statistical computing, and graphical display.[10] Resources are available for learning R, including an online course[11] and introductory book designed for forensic scientists.[12] Another statistical software is JMP®, which warrants consideration due to its interactivity and user-friendly interface. JMP is a commercial product with the price dependent on the number of users.[13] Although the statistical software best suited for each individual laboratory will differ depending on their needs and available funds, it is worthwhile to investigate a variety of options to ensure all resulting validation data is statistically sound and robust for subsequent audit and/or testimony applications.

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