In order to increase the power of discrimination for human identification purposes, a nine-locus short tandem repeat (STR) multiplex, the PowerPlex™ 2.1 System (PowerPlex™ 2.1) has been developed by Promega Corporation. This megaplex system includes the highly polymorphic loci FGA, TPOX, D8S1179, vWA, Penta E, D18S51, D21S11, TH01, and D3S1358 and may be used in combination with the eight-locus STR multiplex, the PowerPlex™ 1.1 system (PowerPlex™ 1.1) which has been previously developed. Three of the loci, TPOX, TH01, and vWA, have been included in both systems for quality control purposes. As with PowerPlex™ 1.1, PowerPlex™ 2.1 is also based on a two-color detection of fluorescent-labeled DNA products amplified by polymerase chain reaction (PCR) and provides a valuable tool for accurate and rapid allele determination. An additional pentanucleotide locus, Penta D, was also tested since it will be part of a new megaplex, PowerPlex™ 16, that will include all loci found in PowerPlex™ 1.1 and PowerPlex™ 2.1 systems. The primer sequences used in the PowerPlex™ 2.1/Penta D system are also presented. To meet the Quality Assurance Standards for Forensic DNA Testing Laboratories, we tested the efficiency and reproducibility of the PowerPlex™ 2.1/Penta D system by several validation studies which were conducted as a joint project among five laboratories. Validation tests included concordance studies, sensitivity and species specificity determination, as well as performance on forensic and environmentally impacted samples. The results produced from these tests demonstrated the consistency and reliability of the PowerPlex™ 2.1/Penta D system. Statistical data obtained from population databases involving major ethnic groups determined independence of loci inheritance and provide several statistical values used for forensic, paternity and other human identification purposes.