A Strategy for Characterization of Single Nucleotide Polymorphisms in a Reference Material

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The advent and adoption of next generation sequencing (NGS) is enabling analysis of single nucleotide polymorphisms (SNPs) at an unprecedented scale, limited primarily by multiplexing during the PCR amplification based enrichment step used for forensic applications. Since only a single nucleotide is assayed, PCR primers may be designed to generate small amplicons, making SNP markers well-suited to forensic DNA typing. Carefully selected panels of SNP markers have been previously established for forensic applications such as one-to-one matching, estimating biogeographical ancestry, and predicting externally observable phenotype [1,2,3,4,5,6]. To support the implementation of SNPs in forensic DNA analysis, NIST will examine the HID-Ion AmpliSeq Identity Panel and the HID-Ion AmpliSeq Ancestry Panel for the Ion Torrent Personal Genome Machine (PGM) and the Illumina ForenSeq DNA Signature kit for the Illumina MiSeq FGx. In total, over 300 SNP markers will be typed with approximately 40 % of loci being represented in more than one multiplex. A strategy combining NGS on orthogonal platforms and Sanger sequencing for characterizing the SNP markers for varying levels of confidence is presented herein. The outcome will be to report only the SNP allele calls, analogous to the mitochondrial sequence variants in NIST Standard Reference Material (SRM) 2392, and *not* the subsequent application of the information (e.g. prediction of ancestry or phenotype). Additionally, we demonstrate the optimization of automated sequencing template preparation using the Ion Chef from Ion Torrent to create an efficient workflow suitable to the forensic DNA laboratory.

Specific Aim: To devise an efficient methodology for characterization of SNP genotype values in a reference material. Using NGS as a primary measurement method, concordance between NGS platforms may be used to obtain high confidence SNP genotype values without exhaustive Sanger sequencing. SNP genotypes discordant across platforms may be disambiguated with Sanger sequencing. Evaluation of Mendelian inheritance patterns in family trios may be used as an additional confirmatory strategy.

Next Generation Sequencing Platforms:
Two platforms with commercial SNP genotyping panels intended for forensic DNA analysis are to be used in this characterization schema: the Ion Torrent Personal Genome Machine and the Illumina MiSeq FGx.

Ion Torrent PGM — Credit: www.lifetechnologies.com
Illumina MiSeq FGx — Credit: www.illumina.com

Ion Torrent PGM

HID-Ion AmpliSeq Identity Panel
90 Identity Informative SNPs (IISNPs) [1,2]
34 Lineage Informative SNPs (LISNPs) [3]

HID-Ion AmpliSeq Ancestry Panel
165 Ancestry Informative SNPs (AISNPs) [4,5]

Illumina MiSeq FGx

ForenSeq Signature Assay Kit - 172 SNP markers:
94 IISNPs [1,2]
56 AISNPs [5]
22 Phenotype Informative SNPs (PISNPs) [6]

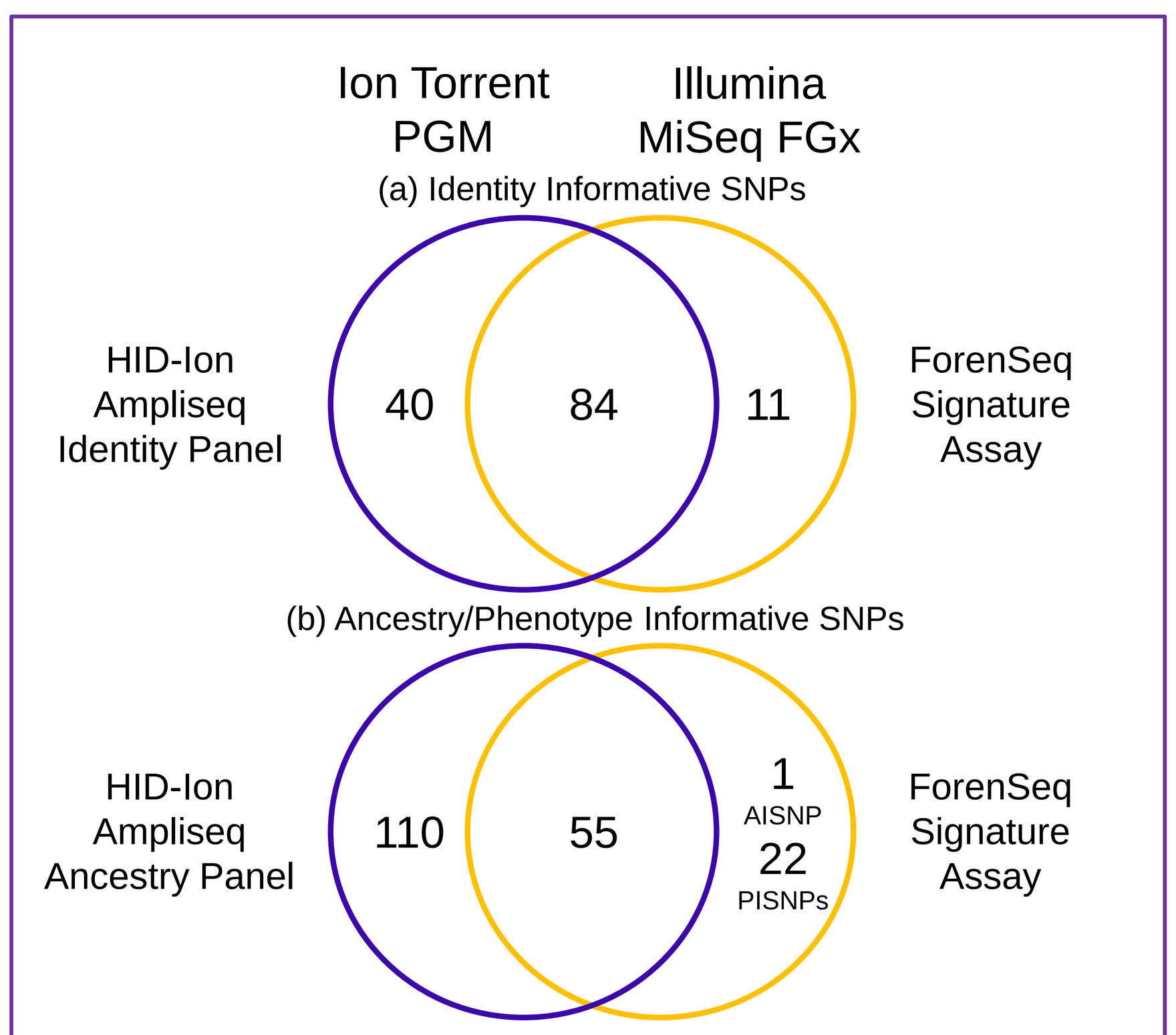

Figure 1: Venn diagrams of the SNP loci in Ion Torrent and Illumina NGS multiplexes with (a) overlapping identity informative markers (IISNPs) and (b) ancestry/phenotype informative markers (AISNPs). The Ion Torrent panels do not include any Phenotype Informative SNPs. There are 139 loci in common (out of a total 323) between the two platforms.

Methods:
Nine single-source DNA samples have been selected as candidate components for a reference material.

Name	Description
RM 1	Female
RM 2	Male
RM 3	Male
RM 4	Female
RM 5	Male
RM 6	Female
RM 7	Family Trio, Son
RM 8	Family Trio, Father
RM 9	Family Trio, Mother

- Typed on PGM Identity Panel & Ancestry Panel
- DNA input ≈ 1 ng
- Three replicate amplifications per sample
- Used library protocol for PGM HID SNPs
- ISP templating & chip loading performed on Ion Chef
- 31 samples in barcoded library pool
  - 9 candidate RMs + 1 control in triplicate
  - One negative control
- Sequenced each panel on one Ion 318 Chip

NIST Levels of Confidence for Certification

	Description	Proposed Method
Certified	NIST has the highest confidence in its accuracy. All known or suspected sources of bias have been investigated or taken into account.	Sanger Sequencing + NGS
Reference	A high-confidence estimate of the true value but where all possible sources of bias have not been fully investigated by NIST.	NGS + NGS Two Platform Concordance
Informational	Data that may be of interest and use to the SRM user, but insufficient information is available to access the confidence of the assignment.	NGS on one platform

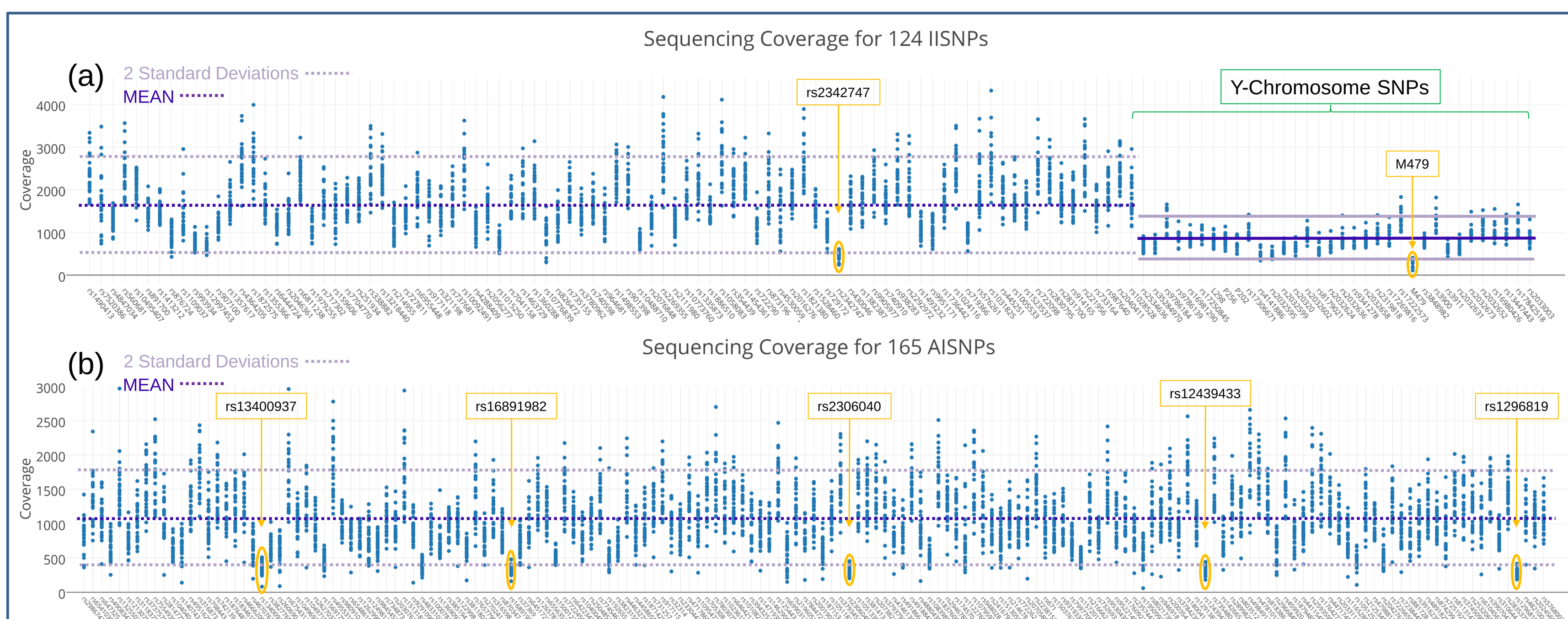

Figure 2: Sequencing coverage of nine candidate reference materials run in triplicate plotted for each SNP locus. (a) Identity Panel SNPs are shown with the 34 Y-chromosome LISNPs at the far right representing five male samples. (b) Ancestry Panel SNPs are shown. Loci with mean coverage below two standard deviations of the mean coverage for all loci are circled.

Indicators of Low Confidence Genotypes

Sequencing coverage: The Ion AmpliSeq Library Preparation for Human Identification Applications (Pub. No. MAN0010640 Rev. A.0) protocol suggests a minimum sequencing coverage of 300 X for autosomal SNPs (150 X for Y-SNPs). To achieve this minimum coverage level for all loci in a panel, an average coverage depth of 738 X is recommended for the Identity Panel and 594 X for the Ancestry Panel. These recommended values allow for locus-to-locus variations in sequencing coverage balance. This translates to a maximum of 77 samples in a library pool when using an Ion 318 Chip for the Identity Panel or 59 samples when running the Ancestry Panel, assuming 80 % chip loading and 60 % usable reads. During NIST's pilot PGM sequencing experiments discussed herein, 31 samples were sequenced concurrently on one 318 chip for each panel. This is roughly half of the maximum number of samples recommended for a 318 chip. In this dataset, two (2) out of 2430 (0.08 %) Identity Panel autosomal SNP genotypes had sequencing coverage below 300 X. The number of Identity Panel Y-chromosome LISNPs with coverage below 150 X was one (1) out of 597 (0.17 %). In the Ancestry Panel data, 90 SNP genotypes out of a total 4455 (2.02 %) were below the suggested minimum 300 X coverage. Developmental validation criteria discussed in the protocol require that the average coverage for a locus should be within two standard deviations of the mean coverage for all loci. Here the mean value of Identity Panel autosomal IISNP loci is 1747.3 X +/- 531.5 X, making the lower threshold for coverage 684.3 X. For Y-chromosome LISNPs, the mean was 849.5 X +/- 236.4 X, making the lower cutoff 376.6 X. For the Ancestry Panel the mean value was 1063.1 X +/- 354.0 X, making the lower threshold for coverage 355.1 X. Higher sequencing coverage was not considered a risk factor for low confidence measurements. Following this principle, one autosomal locus in the Identity Panel (rs2342747 @ 466.1 X) and one Y-chromosome locus (M479 @ 280.2 X) fell below two standard deviations of the mean, while five Ancestry Panel loci were below the criterion: rs1296819 (284.9 X), rs2306040 (323.9 X), rs12439433 (326.0 X), rs16891982 (332.4 X), rs13400937 (346.4 X). Loci with characteristically low coverage are highlighted with orange circles in **Figure 2** and with orange filled cells in **Table 1**.

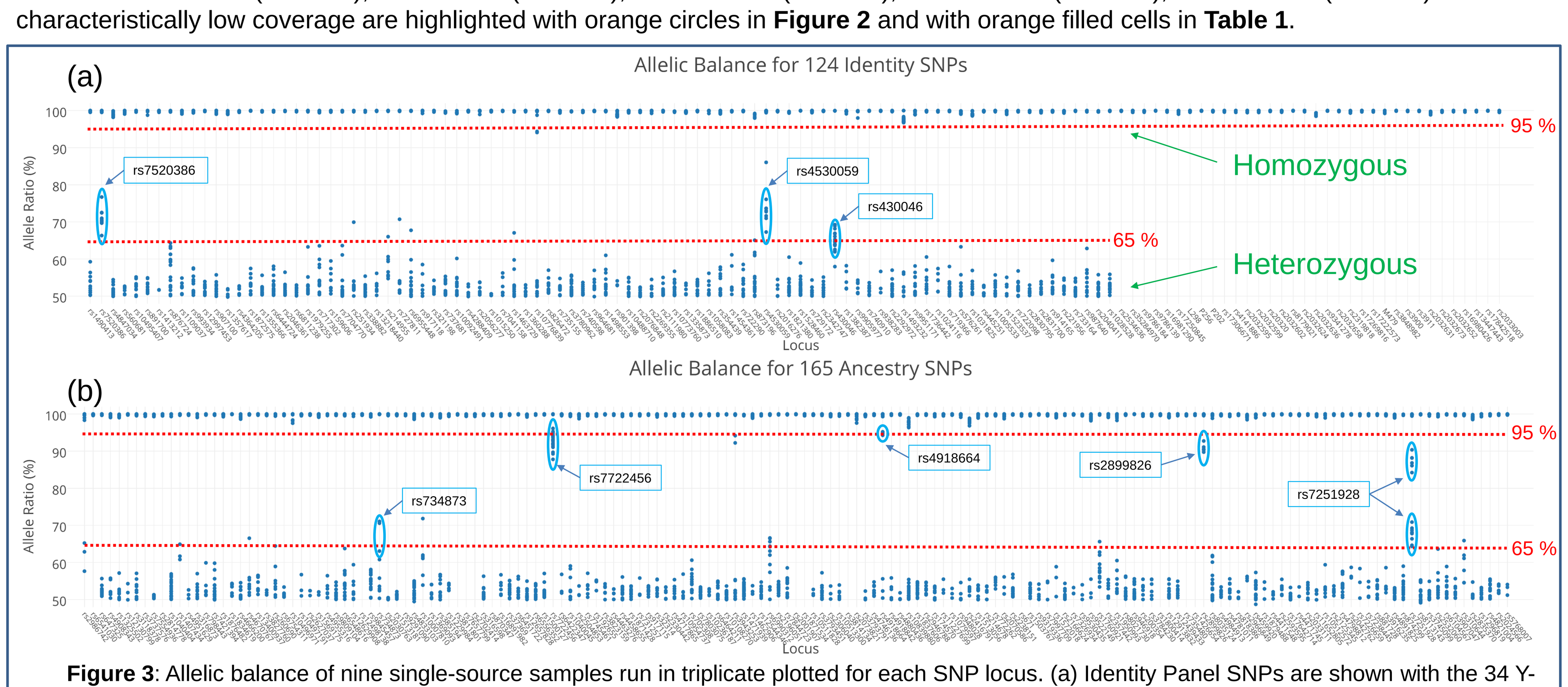

Figure 3: Allelic balance of nine single-source samples run in triplicate plotted for each SNP locus. (a) Identity Panel SNPs are shown with the 34 Y-chromosome LISNPs at the far right, representing five male samples. All Y-chromosome LISNPs are homozygous. (b) Ancestry Panel SNPs are shown. Samples with average allelic balance > 65 % or < 95 % are circled in blue.

Allelic imbalance: heterozygotes should have a sequencing read ratio of 50 % and homozygotes should be 100 %. Deviation from these values may be an indication that a technical issue has occurred with the determination of the genotype. Heterozygotes deviating from the expected 50 % (+/- 15 %) allele ratio may have a SNP underlying a PCR primer binding site which disturbs PCR efficiency or other technical issue such as strand bias or systematic sequencing error. Homozygotes exhibiting a ratio below 100 % (+/- 5 %) could be an indication of systematic sequencing error or bioinformatic sequence alignment issue. Loci with imbalanced allelic ratios are summarized in **Table 3**. A previous study be by Seo *et al*. [7] noted that marker rs4530059 in an earlier version of the Identity Panel displayed allelic imbalance in several heterozygous individuals where the average coverage ratio of A/G was 67.6 %.

Strand bias: defined as the number of plus strand reads divided by the number of minus strand reads, this condition indicates a systematic sequencing error type wherein one strand yields low quality results and is bioinformatically filtered from the final data. Using an arbitrary cutoff of +/- 15 % from the expected ideal ration of 50 % to demark low confidence genotypes, there were five loci in the Identity Panel and nine loci in the Ancestry Panel which yielded at least one data point outside the arbitrary limits of < 35 % and > 65 %. These SNP markers are summarized in **Table 2**. Data points falling outside these arbitrary thresholds may be considered low confidence and will require confirmation with a secondary method.

Discordant replicates: two loci in the Ancestry Panel had discordant genotypes between triplicate data points (see yellow shaded cells in **Tables 1 & 3**). For marker rs3943253 one replicate had a no-call (NN) while the two remaining replicates were homozygous (AA). This marker has an allelic ratio of 93.2 % for the two successfully genotyped replicates and 90.3 for the no-call, however, no quality flag was issued to explain the no-call. All replicates of rs3943253 had some proportion of reads supporting a "G" genotype, which is the alternate allele; the adjacent bases are 5' T and 3' C. The G impurity appears in both forward and reverse reads. No clear explanation is available for this mis-genotype. It may be a case of context-specific sequencing error. A second locus, rs7251928, had one replicate with genotype "CC" and two with "AC". Preliminary data from an alternate platform suggests that the correct call for this locus is "CC". Given that all heterozygotes displayed notable strand bias and allelic imbalance at this locus, resulting genotypes should be considered "very low confidence" until confirmed with an alternate method.

Mendelian inheritance patterns: family trio samples may be used to verify proper genotyping performance. Maternal and Paternal allele inheritance patterns are depicted in **Table 4 below**. Nonconformities in expected inheritance patterns may indicate a technical issue with genotyping a locus. This approach may also be used to identify loci which contain putative SNPs underlying primer binding regions. No inconsistencies in inheritance patterns were identified among family trio samples in the candidate reference materials when evaluated with the Identity Panel or Ancestry Panel. Due to the criteria used in selecting the loci for the Identity Panel (high heterozygosity, low F_{st}) and the Ancestry Panel (low heterozygosity, high F_{st}), there are differences in the percentages of autosomal alleles which may be definitively determined to have originated from one parent versus the other. In the Identity Panel, 38.9 % of loci were informative versus 20.6 % for the Ancestry Panel.

Table 1: Genotypes of nine candidate reference materials characterized for the HID-Ion AmpliSeq Identity Panel (a) and the HID-Ion AmpliSeq Ancestry Panel (b) using the Ion Torrent PGM sequencer.

(a) PGM Identity Panel

Locus	RM 1	RM 2	RM 3	RM 4	RM 5	RM 6	RM 7	RM 8	RM 9
rs1490413	AA	AG	GG	AG	GG	AG	AA	AA	AG
rs7520386	AG	AG	GG	AG	GG	AA	AA	AA	AA
rs4847034	GG	GG	AG	AG	AG	AG	AA	AA	AG
rs560681	AG	AA	GG	AA	GG	AA	AG	GG	AA
rs10495407	GG	AA	AG	GG	AA	AG	AG	GG	AG
rs891700	AG	AA	AA	AG	AG	GG	GG	AG	GG
rs1413212	CT	CC	CC	CC	CC	CC	CT	TT	CC
rs876724	CT	CT	CC	CT	CC	CT	CT	CT	TT
rs1109037	AG	GG	GG	AA	GG	GG	AG	AG	AA
rs993934	GG	AG	AA	AA	AG	AA	AG	AG	AA
rs12997453	AG	AG	GG	GG	GG	AG	AA	AA	AG
rs907100	CG	CG	CG	CG	CC	GG	GG	CG	GG
rs1357617	TT	TT	TT	AA	AT	TT	AT	TT	AA
rs4364205	GT	GT	GG	GG	TT	GG	GG	GT	GG
rs1872575	AG	GG	AG	AG	AA	AG	AA	AA	AA
rs1355366	TT	TT	TT	CT	CT	TT	CC	CT	CC
rs6444724	CT	CT	CT	CC	CT	CT	CT	CT	CC
rs2046361	AT	AT	AT	AT	TT	TT	TT	AT	TT
rs6811238	TT	GT	GG	GT	GG	GG	GG	GT	GT
rs1979255	CG	GG	CG	CG	CG	CG	GG	GG	GG
rs717302	AG	AA	AA	GG	GG	AG	AG	AG	AG
rs159606	AA	AG	GG	AA	AG	AG	AG	GG	GG
rs7704770	AG	GG	GG	AG	AG	AG	AG	AA	AG
rs251934	AA	AA	AG	AG	AG	AG	GG	GG	AG
rs338882	AA	GG	AG	AG	AG	AA	AG	AG	GG
rs13218440	AG	GG	AG	GG	GG	GG	GG	AG	GG
rs214955	CC	CT	CT	CC	CC	CC	CC	CC	CT
rs727811	GG	GG	TT	GT	TT	GT	GG	GT	GG
rs6955448	CC	CC	CC	CT	CT	CT	CT	CT	CT
rs917118	CT	CT	CC	CC	CC	CC	CC	CC	CC
rs321198	CT	CC	CT	TT	CT	CC	CC	CC	CC
rs737681	CT	CT	CC	CT	CT	TT	CT	CT	CT
rs10092491	CT	TT	CC	CT	CC	CT	CT	CC	CT
rs4288409	AA	AC	AC	CC	AA	CC	CC	CC	CC
rs2056277	CC	CC	CC	CT	CC	CC	CC	CT	CC
rs1015250	GG	GG	GG	GG	GG	GG	CG	CC	GG
rs7041158	CC	CC	CC	CC	CT	CT	TT	TT	TT
rs1463729	TT	TT	CC	CT	CT	CT	CT	TT	CT
rs1360288	CT	CC	CC	TT	CT	CC	CC	CC	CT
rs10776839	GG	GG	GG	GT	GT	TT	GG	GT	GT
rs826472	CC	CT	CC	CT	CT	CC	CC	CC	CT
rs735155	CT	CC	CT	CC	CC	TT	CC	CT	CT
rs3780962	AG	AG	AG	AG	AG	AG	AG	GG	AG
rs740598	AG	AA	AA	AA	GG	AA	AG	GG	AG
rs964681	CT	TT	CT	TT	TT	TT	TT	CT	TT
rs1498553	CC	CC	CT	CT	CT	CT	CT	TT	CC
rs901398	TT	TT	CT	TT	CC	CC	TT	TT	TT
rs10488710	CC	GG	CG	CG	CC	CC	CG	CG	GG
rs2076848	TT	AA	AT	AA	AT	AA	TT	TT	AT
rs2269355	CG	GG	GG	CC	CG	CC	CC	CC	CC
rs2111980	TT	CT	TT	CT	CT	CC	CC	CT	CC
rs10773760	AG	AG	AG	GG	AG	AG	AA	AG	AG
rs1335873	AT	TT	AA	AA	AA	AA	AA	AT	AA
rs1886510	AG	GG	GG	AG	AG	GG	AG	GG	AG
rs1058083	GG	AG	GG	GG	GG	GG	AG	GG	AG
rs354439	AT	AT	TT	AA	AT	AT	TT	TT	TT
rs1454361	AT	TT	AA	TT	AA	AT	TT	TT	TT
rs722290	CG	CC	GG	CG	CC	CG	CG	GG	CG
rs873196	CT	TT	CT	CT	CT	CT	CT	CT	TT
rs4530059	GG	AG	AA	GG	GG	GG	AG	AG	GG
rs2016276	TT	TT	TT	TT	TT	TT	CT	TT	CT
rs1821380	CG	GG	GG	CG	CG	CG	CG	GG	CG
rs1528460	TT	CT	CT	TT	CT	CT	CT	CT	CT
rs729172	GT	TT	GG	GG	GT	GG	GG	GT	GG
rs2342747	AG	GG	AA	AA	GG	AG	AG	AA	AG
rs430046	CT	CT	CC	TT	CT	CT	CT	CT	CT
rs1382387	CC	AA	AA	AA	AA	AC	AA	AC	AA
rs9905977	AG	GG	AG	GG	AG	GG	GG	GG	GG
rs740910	AA	AG	AA	AG	AG	AG	AA	AA	AA
rs938283	TT	CT	TT	TT	TT	TT	TT	TT	TT
rs2292972	CC	CT	TT	CT	CT	CC	CT	CT	CT
rs1493232	AA	AC	CC	AA	AC	AA	AA	AA	AA
rs9951171	AG	GG	GG	AG	AG	AG	AG	AA	AG
rs1736442	CT	CT	CC	CC	TT	CT	CT	CC	TT
rs1024116	CT	TT	CC	CT	TT	TT	TT	TT	TT
rs719366	AA	AG	AG	AA	AG	AG	AG	AG	AA
rs576261	AC	AC	AC	AA	AA	AC	CC	CC	AC
rs1031825	AC	AC	CC	CC	AA	AC	CC	CC	CC
rs445251	CG	CC	GG	GG	CG	CC	GG	CG	GG
rs1005533	GG	GG	GG	AA	AA	AG	AG	AG	AG
rs1523537	TT	CT	CC	CC	TT	CC	CC	CT	CC
rs722098	AA	AA	AG	AA	AG	AG	AA	AG	AA
rs2830795	AA	GG	AA	AA	AG	AA	AG	AG	AG
rs2831700	AG	AG	AG	AA	AA	GG	GG	GG	GG
rs914165	AA	GG	AG	GG	GG	AG	GG	AG	AG
rs221956	CT	TT	CT	CC	CT	CC	CT	CC	TT
rs733164	AG	GG	GG	AG	AG	AG	GG	AG	AG
rs987640	AT	TT	AT	TT	AA	AT	TT	TT	AT
rs2040411	AG	AA	AG	AG	AA	AA	AG	AG	AG
rs1028528	GG	AA	AG	AA	AG	AA	AG	AA	AG
Y-chromosome markers									
rs2534636	-	C	C	-	C	-	C	C	-
rs35284970	-	C	C	-	C	-	C	C	-
rs9786184	-	C	C	-	C	-	C	C	-
rs9786139	-	G	G	-	G	-	G	G	-
rs16981290	-	C	C	-	C	-	C	C	-
rs17250845	-	C	G	-	G	-	C	C	-
L298	-	T	T	-	T	-	T	T	-
P256	-	G	G	-	G	-	G	G	-
P202	-	T	T	-	T	-	T	T	-
rs17306671	-	A	T	-	A	-	A	A	-
rs4141886	-	A	A	-	A	-	A	A	-
rs2032595	-	T	T	-	T	-	T	T	-
rs2032599	-	T	T	-	T	-	T	T	-
rs20320	-	G	G	-	G	-	G	G	-
rs2032602	-	T	T	-	T	-	T	T	-
rs8179021	-	C	C	-	C	-	C	C	-
rs2032624	-	A	A	-	A	-	A	A	-
rs2032636	-	G	G	-	G	-	G	G	-
rs9341278	-	G	G	-	G	-	G	G	-
rs2032658	-	A	A	-	A	-	A	A	-
rs2319818	-	G	G	-	A	-	G	G	-
rs17269816	-	C	C	-	C	-	C	C	-
rs17222573	-	A	A	-	A	-	A	A	-
M479	-	C	C	-	C	-	C	C	-
rs3848982	-	C	C	-	C	-	C	C	-
rs3900	-	C	G	-	C	-	C	C	-
rs3911	-	A	A	-	A	-	A	A	-
rs2032631	-	G	G	-	G	-	G	G	-
rs2032673	-	T	T	-	T	-	T	T	-
rs2032652	-	T	T	-	T	-	T	T	-
rs16980426	-	T	T	-	T	-	T	T	-
rs13447443	-	A	A	-	A	-	A	A	-
rs17842518	-	G	G	-	G	-	G	G	-
rs2033003	-	A	C	-	A	-	A	A	-

(b) PGM Ancestry Panel

Locus	RM 1	RM 2	RM 3	RM 4	RM 5	RM 6	RM 7	RM 8	RM 9
rs2986742	CC	TT	TT	TT	TT	TT	TT	TT	CT
rs6541030	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs647325	AA	AA	AG	AA	AG	AA	AA	AG	AG
rs4908343	AG	AA	GG	AA	AA	AG	AA	AA	AA
rs1325502	GG	GG	AA	AG	AG	AG	GG	GG	GG
rs12130799	AA	AA	AA	AA	AA	AA	AA	AG	AA
rs3118378	AA	GG	AA	AG	AA	GG	AA	AG	AG
rs3737576	TT	CC	TT	TT	TT	TT	TT	TT	TT
rs7554936	CT	TT	TT	CT	TT	CT	TT	TT	TT
rs2814778	TT	TT	TT	TT	TT	TT	TT	TT	TT
rs1040404	AG	AG	AG	AG	AG	GG	AG	AG	AA
rs1407434	GG	AG	GG	GG	GG	GG	GG	GG	GG
rs4951629	TT	CT	TT	TT	TT	TT	TT	TT	TT
rs316873	CT	CT	TT	CC	CC	CT	CC	CC	CC
rs798443	AA	AA	AA	AG	AA	AG	AA	AA	AA
rs7421394	AA	AG	GG	AG	AA	AG	AG	AG	GG
rs1876482	GG	GG	AA	GG	GG	GG	GG	GG	GG
rs1834619	GG	AG	GG	GG	GG	GG	GG	GG	GG
rs4666200	AG	AG	AA	AA	AG	AA	GG	AG	AG
rs4670767	GT	GG	GT	GT	GG	GG	GG	GG	GG
rs13400937	TT	TT	GT	TT	GT	TT	TT	TT	GT
rs3827760	AA	AA	AG	AA	AA	AA	AA	AA	AA
rs260690	AA	AA	AC	AC	AA	AC	AA	AA	AA
rs6754311	TT	CT	CC	CT	TT	TT	CC	CC	CC
rs10496971	TT	TT	GG	TT	TT	TT	TT	TT	TT
rs10497191	CC	CC	CC	CT	CC	CC	CC	CC	CC
rs2627037	GG	AG	GG	GG	GG	GG	GG	GG	GG
rs1569175	CC	CC	CC	CC	CC	CT	CC	CC	CC
rs4955316	GT	GT	TT	TT	GT	TT	TT	TT	TT
rs9809104	TT	CT	TT	TT	TT	TT	CT	TT	CT
rs6548616	TT	CT	CT	TT	CC	TT	CT	CT	CT
rs12629908	GG	AG	AG	GG	GG	GG	GG	GG	AG
rs12498138	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs9845457	AG	GG	AA	AG	AG	AA	AG	AG	AA
rs734873	GG	GG	GG	GG	GG	GG	AG	AG	AG
rs2030763	GG	AG	GG	GG	GG	AG	GG	GG	GG
rs1513181	AG	GG	GG	GG	AG	GG	GG	AG	GG
rs9291090	AA	AA	AA	AA	AA	AA	AA	AA	AA
rs4833103	AC	AC	CC	AC	AC	AC	CC	CC	CC
rs10007810	GG	AG	AG	GG	GG	GG	GG	GG	GG
rs1369093	TT	TT	CT	TT	TT	TT	TT	CT	TT
rs385194	GG	AG	AG	AA	GG	GG	AA	AA	AG
rs1229984	CC	CT	CT	CC	CC	CC	CC	CC	CC
rs3811801	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs7657799	TT	TT	TT	TT	TT	TT	TT	TT	TT
rs2702414	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs316598	TT	TT	CT	TT	CT	TT	CT	CT	CT
rs870347	AA	AA	AA	AA	AA	AA	AA	AA	AA
rs16891982	GG	CG	CC	CG	GG	GG	GG	GG	GG
rs37369	CC	CT	CT	CT	CC	TT	CT	CC	CT
rs6451722	GG	GG	AG	AG	GG	AG	GG	GG	GG
rs12657828	AA	GG	AG	AG	AA	AG	AG	AA	AG
rs6556352	CC	CT	CT	CT	CT	CC	CT	CC	TT
rs1500127	CT	CT	CT	CC	CT	CC	CC	CC	CC
rs7722456	TT	TT	CC	TT	TT	TT	TT	TT	TT
rs6422347	TT	CT	TT	TT	TT	TT	TT	TT	TT
rs1040045	AG	AA	AA	AG	AA	AA	AA	AG	AA
rs2504853	CT	CC	CC	TT	TT	CC	TT	TT	TT
rs7745461	AG	AG	GG	GG	GG	GG	GG	GG	GG
rs192655	AA	AA	AA	AG	AA	AA	AA	AA	AA
rs3823159	AA	AG	AG	AA	AA	AA	AA	AA	AA
rs4463276	GG	AA	AA	AA	AG	GG	AG	AG	GG
rs4458655	TT	CT	CT	TT	CT	TT	CT	TT	TT
rs1871428	AG	AG	AA	AG	GG	AG	GG	GG	GG
rs731257	GG	AA	AG	GG	GG	GG	GG	GG	GG
rs917115	CT	CC	CC	TT	TT	CC	CT	CC	CT
rs32314	CT	CT	CC	TT	CT	TT	TT	TT	TT
rs2330442	GG	AA	AA	AA	GG	AA	AG	AG	AA
rs4717865	GG	GG	GG	GG	GG	GG	AG	AG	AG
rs10954737	TT	CT	TT	TT	TT	CT	TT	TT	TT
rs705308	CC	AC	AC	CC	CC	AC	AA	AC	AC
rs7803075	AG	AA	AA	GG	AG	GG	AG	GG	AG
rs10236187	AA	CC	AA	AA	AA	AA	AC	AC	AA
rs6464211	CC	CT	CT	CC	CC	CT	CC	CT	CC
rs10108270	AC	AC	CC	AC	CC	AC	AC	AC	AC
rs3943253	AA	AG	AA	AA	AG	AG	AA	AA	AG
rs1471939	CT	CT	CT	TT	CT	CT	CT	CT	CT
rs1462906	CT	CC	CC	CC	CC	CC	CC	CC	CC
rs12544346	AA	AG	GG	GG	AG	AA	AG	AG	AG
rs6990312	GT	GG	TT	GG	GT	GG	GT	GT	GG
rs2196051	AG	AG	GG	AA	AA	AG	AG	AG	AA
rs7844723	CT	CT	CC	CC	TT	CT	CT	CC	CT
rs2001907	CC	CC	CC	CC	CC	CC	CC	CC	CC
rs1871534	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs10511828	TT	TT	TT	TT	TT	TT	TT	TT	CT
rs3793451	CT	CC	CC	CT	CC	CC	CT	CC	CT
rs2306040	TT	TT	TT	TT	TT	TT	TT	TT	CT
rs10513300	CT	TT	CT	TT	TT	TT	TT	TT	TT
rs3814134	AA	AA	AA	AA	AA	AA	AA	AA	AA
rs2073821	CC	TT	CC	CC	CC	CC	CC	CC	CC
rs3793791	TT	TT	CT	TT	TT	TT	TT	TT	TT
rs4746136	AA	AA	AG	AG	GG	GG	GG	GG	AG
rs4918664	AA	GG	GG	AA	AA	AG	AA	AA	AA
rs4918842	TT	TT	CC	TT	CT	CT	TT	TT	TT
rs4880436	CC	CC	CC	CC	CC	CT	CC	CC	CC
rs10839880	TT	TT	CT	CT	CT	TT	TT	CT	CT
rs1837606	CT	CT	CT	CT	CC	TT	CT	TT	CT
rs2946788	GT	GT	GT	GG	TT	GT	TT	TT	TT
rs174570	CC	CT	TT	CT	CC	CT	CC	CC	CT
rs11227699	AA	AG	GG	GG	GG	GG	GG	GG	GG
rs1079597	CC	TT	CC	CC	CC	CT	CC	CC	CC
rs948028	AA	AC	AA	AA	AA	AA	AC	CC	AA
rs2416791	GG	GG	GG	AG	GG	GG	GG	GG	AG
rs1513056	GG	AA	AA	AG	GG	AG	AG	AG	GG
rs214678	TT	TT	TT	TT	TT	CT	TT	CT	TT
rs772262	GG	GG	GG	GG	GG	AG	GG	AG	GG
rs2070586	GG	GG	AG	AA	AA	GG	GG	GG	GG
rs2238151	CT	CC	TT	CT	CT	TT	CT	CT	TT
rs671	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs1503767	TT	TT	GT	GT	TT	TT	TT	TT	GT
rs9319336	TT	CT	CT	TT	TT	TT	TT	TT	TT
rs7997709	TT	CC	TT	CT	TT	TT	TT	TT	TT
rs1572018	CC	CC	TT	CC	CC	CC	CT	TT	CC
rs2166624	GG	AA	GG	AG	GG	AG	GG	AG	AG
rs7326934	GG	GG	CG	GG	GG	GG	GG	GG	GG
rs9530435	CT	CC	CC	TT	CC	CC	CC	CC	CC
rs9522149	TT	TT	TT	CC	TT	CT	CC	CC	CC
rs1760921	TT	CT	CT	TT	TT	TT	CT	CT	CT
rs2357442	AA	AA	AA	AC	AA	CC	AA	AC	AA
rs1950993	GT	TT	TT	GT	TT	GG	TT	TT	GT
rs8021730	GG	GG	GT	GT	GG	GG	GT	GT	GG
rs946918	GG	GT	GG	GT	GT	GT	GT	GG	GT
rs200354	GT	GT	TT	GG	GG	GG	GG	GG	GG
rs3784230	AA	AG	AA	AA	AA	GG	AA	AG	AA
rs1800414	TT	TT	TT	TT	TT	TT	TT	TT	TT
rs12913832	GG	AG	AA	AG	AG	GG	GG	GG	AG
rs12439433	AA	AG	AA	AA	AA	AA	AG	AA	AG
rs735480	TT	TT	CC	TT	TT	TT	TT	TT	TT
rs1426654	AA	AA	GG	AA	AA	AA	AA	AA	AA
rs2899826	AA	GG	GG	AA	AA	AA	AA	AA	AA
rs8035124	AA	CC	AC	AA	AA	AC	AC	AC	AC
rs4984913	AA	AA	GG	AG	AG	AG	GG	GG	AG
rs4781011	GG	GT	TT	GG	GT	GG	GT	GT	GG
rs818386	CC	TT	CC	CC	CC	CT	CC	CC	CC
rs2966849	GG	GG	GG	GG	GG	AG	GG	GG	GG
rs459920	CT	CT	TT	CT	CT	CT	CT	CC	TT
rs1879488	CC	CC	CC	AC	CC	CC	CC	CC	CC
rs4411548	CC	CC	CC	CC	TT	CC	CT	CT	CC
rs2593595	AA	AA	AA	AG	AA	AA	AA	AA	AG
rs17642714	AT	AA	AA	AA	AA	AA	AT	AA	AT
rs4471745	GG	GG	AA	GG	AG	GG	GG	GG	GG
rs2033111	AA	AG	AA	AG	AG	AA	AA	AA	AA
rs11652805	TT	CT	CT	TT	TT	TT	TT	TT	TT
rs10512572	GG	AG	AG	GG	GG	GG	GG	GG	GG
rs2125345	CC	TT	TT	CC	CT	TT	TT	TT	CT
rs4798812	GG	AG	GG	AG	AG	GG	AA	AG	AA
rs2042762	TT	CT	TT	TT	TT	TT	TT	TT	TT
rs7226659	GG	GT	TT	GG	GG	GG	GG	GG	GG
rs7238445	AG	GG	GG	GG	AG	GG	GG	GG	GG
rs881728	CC	CC	AC	AC	CC	CC	AC	AC	CC
rs3916235	CC	CC	CC	CC	CC	CC	CT	CT	CC
rs4891825	AA	AA	AA	AA	AG	AA	AG	AG	AA
rs874299	CT	CT	TT	CT	CT	CT	CC	CC	CC
rs7251928	AA	AC	AC	AA	AA	CC	AC	AC	AA
rs8113143	AC	AC	AC	AC	AC	AC	CC	CC	CC
rs3745099	AA	AG	AG	AA	AA	AG	AA	AG	AG
rs2532060	CT	CC	CC	CC	CC	TT	CT	CT	CT
rs6104567	TT	GG	TT	GT	GT	GT	TT	GT	TT
rs3907047	TT	CT	TT	TT	TT	TT	CT	TT	CT
rs310644	TT	TT	CC	TT	TT	TT	CT	CT	TT
rs2835370	TT	TT	TT	TT	TT	TT	CT	CT	TT
rs1296819	CC	CC	AC	CC	AC	CC	AA	AC	AC
rs4821004	CT	CT	CT	CC	CT	CC	CC	CC	CC
rs2024566	AG	AA	AA	AA	AA	AG	AA	AA	AA
rs5768007	CC	CT	CC	CC	CC	CC	CC	CC	CC

Legend	
(yellow)	Discordant Replicate
(orange)	Coverage < 2 SD of the mean
(blue)	Allelic Imbalance

Table 2: Average strand bias (% positive strand) for loci with at least one data point < 35 % or > 65 %.

Identity Panel		Ancestry Panel	
M479 *	17.1 +/- 2.4	rs1040045	39.3 +/- 6.3
rs430046 **	70.1 +/- 11.9	rs1760921	73.6 +/- 8.5
rs1463729	63.8 +/- 2.5	rs1871428	35.5 +/- 2.6
rs2111980	39.3 +/- 3.5	rs1871534	36.5 +/- 2.8
rs4364205	63.0 +/- 2.5	rs2986742	46.7 +/- 8.9
		rs6990312	38.3 +/- 10.2
		rs7251928 **	40.1 +/- 13.3
* Low coverage		rs9845457	45.2 +/- 5.9
** Allelic imbalance		rs12629908	41.6 +/- 4.6

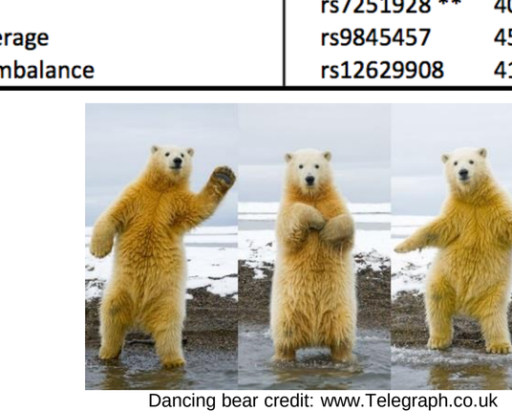

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Table 3: Genotypes and allelic ratios of loci exhibiting allelic imbalance (shaded blue) for the HID-Ion AmpliSeq Identity Panel (a) and HID-Ion AmpliSeq Ancestry Panel (b). * Strand bias observed for this locus.

(a)

Locus	RM 1	RM 2	RM 3	RM 4	RM 5	RM 6	RM 7	RM 8	RM 9
rs7520386	AG	AG	GG	AG	GG	AA	AA	AA	AA
Ratio (%)	71.6 +/- 1.5	70.3 +/- 0.7	99.9 +/- 0.1	71.2 +/- 5.2	99.8 +/- 0.3	99.8 +/- 0.1	99.9 +/- 0.0	99.8 +/- 0.0	99.9 +/- 0.0
rs4530059	GG	AG	AA	GG	GG	GG	AG	AG	GG
Ratio (%)	99.9 +/- 0.1	70.4 +/- 2.9	99.5 +/- 0.1	99.8 +/- 0.2	99.9 +/- 0.1	100.0 +/- 0.0	73.4 +/- 0.5	77.9 +/- 7.4	99.9 +/- 0.1
rs430046 *	CT	CT	CC	TT	CT	CT	CT	CT	CT
Ratio (%)	66.7 +/- 3.6	64.2 +/- 0.4	100.0 +/- 0.0	99.9 +/- 0.0	64.4 +/- 2.3	63.7 +/- 1.6	65.9 +/- 1.7	63.9 +/- 5.5	65.8 +/- 3.7

(b)

Locus	RM 1	RM 2	RM 3	RM 4	RM 5	RM 6	RM 7	RM 8	RM 9
rs734873	GG	GG	GG	GG	GG	GG	AG	AG	AG
Ratio (%)	100.0 +/- 0.0	100.0 +/- 0.1	100.0 +/- 0.0	100.0 +/- 0.1	100.0 +/- 0.0	99.9 +/- 0.0	55.4 +/- 4.7	68.2 +/- 4.5	53.2 +/- 2.7
rs7722456	TT	TT	CC	TT	TT	TT	TT	TT	TT
Ratio (%)	93.5 +/- 1.6	95.2 +/- 1.0	100.0 +/- 0.1	92.8 +/- 0.9	91.6 +/- 0.5	93.3 +/- 1.7	91.7 +/- 2.2	91.3 +/- 3.0	91.3 +/- 1.4
rs3943253	AA	AG	AA	AA	AG	AG	AA	AA	AG
Ratio (%)	99.9 +/- 0.1	54.3 +/- 1.6	93.2 +/- 1.4	99.9 +/- 0.1	51.0 +/- 1.3	51.2 +/- 1.0	99.8 +/- 0.1	99.9 +/- 0.1	51.6 +/- 0.6
rs4918664	AA	GG	GG	AA	AA	AG	AA	AA	AA
Ratio (%)	99.7 +/- 0.4	94.8 +/- 0.5	99.8 +/- 0.2	99.8 +/- 0.2	100.0 +/- 0.1	52.8 +/- 2.6	99.9 +/- 0.1	99.9 +/- 0.0	100.0 +/- 0.0
rs2899826	AA	GG	GG	AA	AA	AA	AA	AA	AA
Ratio (%)	99.8 +/- 0.1	91.3 +/- 0.7	90.4 +/- 0.7	99.8 +/- 0.2	99.8 +/- 0.1	99.9 +/- 0.1	99.9 +/- 0.1	99.9 +/- 0.1	99.9 +/- 0.1
rs7251928 *	AA	AC	AC	AA	AA	CC	AC	AC	AA
Ratio (%)	99.7 +/- 0.2	86.2 +/- 2.0	67.7 +/- 3.3	99.9 +/- 0.1	99.8 +/- 0.1	88.4 +/- 1.8	67.5 +/- 1.0	67.5 +/- 2.5	99.8 +/- 0.1

Discussion:
The majority of SNP loci in the Ion Torrent Identity and Ancestry Panels satisfy proposed requirements for an "informational" classification in a NIST SRM. A small number of loci in these Panels were found to have characteristics signifying potential technical sources of error leading to uncertainty in the measurement. These markers require additional characterization before achieving any status. Confirmation of SNP genotypes with a second NGS method would satisfy the proposed requirements for a "reference value" in a NIST SRM. Final confirmation with Sanger-type sequencing is required to achieve a "certified" value under the proposed framework.

Future Directions:
There are 139 loci which are present in both the HID-Ion AmpliSeq Panels and the Illumina ForenSeq Signature Prep kit. Concordance between the two platforms would qualify those loci as "reference values" for a NIST SRM. Discordant loci will require disambiguation using an alternate method such as Sanger sequencing in order to achieve any status for a NIST SRM. NIST intends to characterize the SNP loci in the Illumina ForenSeq Signature kit. NIST will consider the needs of the forensic community in terms of required levels of confidence in genotypes when prioritizing tasks leading to the potential development of a reference material for SNP genotyping.

References:
1. Musgrave-Brown E., Ballard D., Balogh, K. *et al.*: Forensic validation of the SNP*for*ID 52-plex assay. *Forensic Science International: Genetics*. 2007; 1, 186–190.
2. Pakstis A, Speed W, Fang R, Hyland F, Furtado M, Kidd J, and Kidd K: SNPs for a universal individual identification panel. *Human Genetics*. 2010; 127(3), 315–324.
3. Karafet TM, Mendez FL, Meilerman M, Underhill P, Zegura S, and Hammer M.: New binary polymorphisms reshape and increase resolution of the human Y chromosomal haplogroup tree. *Genome Research*. 2008; 18(5), 830-838.
4. Nassir R, Kosoy R, Tian C, White PA, Butler LM, Silva G, Kittles R, Alarcon-Riquelme ME, Gregersen PK, Belmont JW, De La Vega FM and Seldin MF: An ancestry informative marker set for determining continental origin: validation and extension using human genome diversity panels. *BMC Genetics*. 2009; 10(1), 39.
5. Kidd K, Speed W, Pakstis A, Furtado M, Fang R, Madbouly A, Maiers M, Middha M, Friedlander F, and Kidd J: Progress toward and efficient panel of SNPs for ancestry inference. *Forensic Science International: Genetics*. 2014; 10, 23-32.
6. Walsh S, Liu F, Wollstein A, Kovatsi L, Ralf A, Kosiniak-Kamysz A, Branicki W and Kayser M: The HIrisPlex system for simultaneous prediction of hair and eye colour from DNA. *Forensic Science International: Genetics*. 2013; 7, 98–115.
7. Seo S, King J, Warhsauer D, Davis C, Ge J, and Budowle B: Single nucleotide polymorphism typing with massively parallel sequencing for human identification. *Int J Legal Med*. 2013; 127, 1079-1086.

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Table 4: Mendelian inheritance patterns in Family trio samples for the HID-Ion AmpliSeq Identity Panel SNPs (a) and HID-Ion AmpliSeq Ancestry Panel SNPs (b).

Legend	
(blue)	Paternal copy
(magenta)	Maternal copy
(grey)	Ambiguous
(white)	Not inherited

Poster available for download from STRBase
http://www.cstl.nist.gov/biotech/strbase/pub_pres/KieslerISFG2015poster.pdf