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Pixels v. Nucleotides? Comparing camera and genetic techniques to assess demographics of San Francisco Bay Area river otters

submitted to:
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Abstract

North American river otters (*Lontra canadensis*) have been observed in Marin County with some frequency from the early 2000s yet remained poorly documented, and were listed as ''non-occurring'' in Marin County by the California Department of Fish and Wildlife until 2019. The 2007 Cosco Busan incident revealed the susceptibility of San Francisco Bay Area (SFBA) river otters to oil exposure through foraging on oiled prey species, which has been shown to have long-term impacts on their health and reproductive success. However, the actual impact of the *Cosco Busan* spill remains unknown because there were no pre-spill abundance estimates, or even presence/absence data, for any locations in the region.

Cost-effective and reliable methods for estimating SFBA river otter abundance and distribution are needed, particularly in areas where otter presence/absence is changing or unknown, in order to document baseline information and monitor future changes. Both noninvasive camera and genetic techniques are promising approaches for generating qualitative and quantitative information on distribution and abundance of river otters. This community-science driven study was able to: 1) Estimate minimum population abundance and distribution of river otters at select sites in the SFBA using camera trapping; 2) Use genetic analyses to identify individuals for minimum abundance estimation; 3) Compare minimum population estimates derived from camera trapping and genetic analyses; and 4) Compare two different genetic techniques for cost, speed, and efficacy in individual identification and for potential use in tracking individual movements and dispersal. Our results suggest that camera trapping, periodically supplemented by genetic analysis, can provide reliable information on river otter abundance and distribution.

Background Information

Absent for decades in much of the San Francisco Bay Area (SFBA), North American river otters (*Lontra canadensis*) were observed in Marin County with some frequency from the early 2000s. However, the distribution and abundance of river otters remained poorly documented at any agency level (Bouley et al., 2015). In fact, river otters were listed as ''non-occurring'' in Marin County by the California Department of Fish and Wildlife (CDFW) (Zeiner et al., 1988) up until

2019. At that time, CDFW published a new range map (CDFW 2019) that incorporated a dataset submitted by River Otter Ecology Project (ROEP) for the purpose of revising the range map to show river otters were present in the SFBA.

The 2007 *Cosco Busan* oil spill highlighted this critical data gap when river otters in Rodeo Lagoon were observed feeding on oiled birds (Salman, 2007) and oil residues were detected in otter feces (*Cosco Busan* DARP, 2012). River otters are mesocarnivores that prey on an array of species such as native and non-native freshwater, anadromous, and marine fishes, waterbirds, crustaceans, and amphibians (Melquist et al., 2003; Penland and Black, 2009; Boone, 2013; Cosby, 2013; Crowley et al., 2013; Garwood et al., 2013; ROEP, unpublished data). The *Cosco Busan* incident revealed the susceptibility of river otters to oil exposure through foraging on oiled prey species, which has been shown to have long-term impacts on their health and reproductive success (Bowyer et al., 2003). Prior studies documenting effects of oil spills on otters confirm they are a keystone species for the marine-land interface and a sentinel species for monitoring levels of environmental contamination in coastal habitats (Bowyer et al., 2003). River otters are relatively long-lived (≤13 years in the wild; Docktor et al. 1987), are sexually mature by 2 years (Hamilton and Eadie 1964, Docktor et al. 1987), and do not migrate or hibernate (Melquist and Hornocker 1983, Bowyer et al. 1995) potentially exposing them to year-round localized sources of pollution, including oil (Bowyer et al., 2003).

Unfortunately, the actual impact of the *Cosco Busan* spill on SFBA river otters remains unknown because there were no pre-spill abundance estimates (or even presence/absence data) for any locations in the region. Without a baseline in the Bay Area – a baseline being the priority outcome of the OWCN-supported work described in this report – agencies will never understand the full impact of catastrophic events such as spills on SFBA river otter populations, or be prepared to take the necessary actions to remediate these impacts.

During 2012, ROEP was launched to address this deficit of data on river otter populations in coastal, bay, river, and lake habitats of the SFBA. During February of 2012, ROEP started a community-science initiative called ‘‘Otter Spotter’’ to solicit structured data from the public on river otter sightings from the 9-county region surrounding San Francisco Bay. In tandem with the launch of our web-based portal, we began year-round field investigations at key locations along a 197 km stretch of coastline and stream channels, spanning from San Francisco north to Tomales Bay and inland on Lagunitas Creek and its tributaries and reservoirs. River Otter Ecology Project’s long-term monitoring project has documented the steady recovery of river otter populations in most of these study areas, with some exceptions (Carroll et al., 2020). It is our belief that the populations are still in recovery and now inhabit areas that have been and may be again impacted by oil spills (Figure 1).

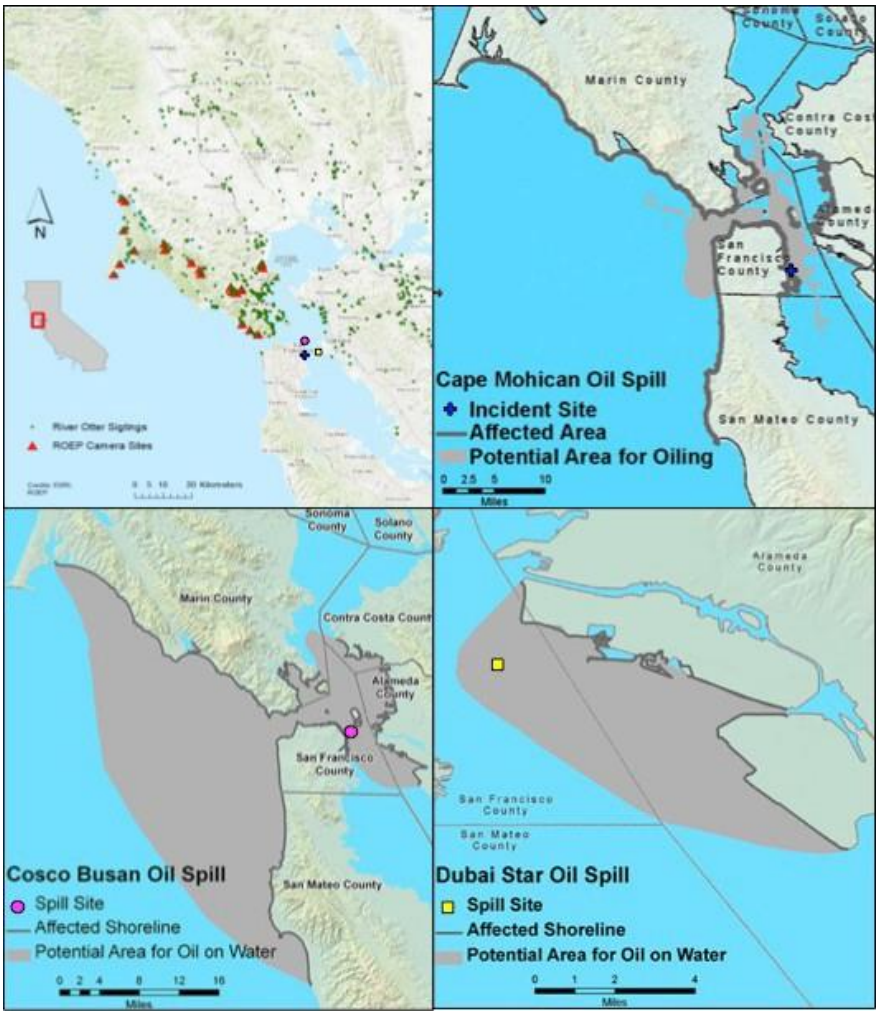

Figure 1. Map of the San Francisco Bay Area (SFBA) with River Otter Ecology Project Otter Spotter reports and focal study sites (2012 and 2013) demonstrating potential overlap with major oil spill events in the SFBA modified from California Department Fish and Wildlife Office of Spill Prevention and Response Natural Resource Damage Assessment Program maps.

An understanding of otter abundance, distribution and population differentiation is necessary to understand current demography and distribution, prepare for future spills, and assess impacts. Specifically, cost-effective and reliable methods for estimating abundance and distribution are needed, particularly in areas where otter presence/absence is changing or unknown, in order to document baseline information and monitor future changes.

Noninvasive camera trapping is a commonly used technique to study elusive, wide-ranging carnivores, including river otters (Olson et al., 2005; Olson et al., 2008; Stevens and Serfass, 2008; Green et al., 2015; Day et al., 2016; Wagnon and Serfass, 2016). However, North American river otters often lack distinctive markings, making it difficult to identify individual

otters from photographs and videos (Reed-Smith, 2012) and cameras don't always capture individuals visiting the site (Stevens et al., 2004).
Both noninvasive camera and genetic techniques are promising approaches for generating qualitative and quantitative information on distribution and abundance of river otters. Integrated abundance estimates from both techniques likely will provide a more repeatable and accurate assessment of otter presence and abundance in the SFBA and beyond, their vulnerability to spills, and the potential investment of resources for oil spill response.

Hypotheses and Specific Aims
The specific aims of our study were to: 1) Estimate minimum population abundance and distribution of river otters at select sites in the SFBA using noninvasive camera-trapping techniques; 2) Use genetic analyses to evaluate population differentiation of river otters in the SFBA and genetically identify individuals, then use capture-recapture analyses for abundance estimation; 3) Compare minimum population abundance estimates derived from camera-trapping and genetic analyses; and 4) Determine whether a consistent correction factor can be used for data collected from a different set of sites during Year 1 and Year 2.

Experimental Design
We originally proposed a two-year study, with Year 1 a pilot investigation to estimate abundance and distribution of select river otter populations in Marin County through noninvasive camera-trapping techniques and fecal-sample genetic analyses. The efficacy of the two methods would then be compared, and during Year 2, the methods could then be evaluated, refined, and applied to additional areas of San Francisco Bay (e.g., Martinez, Richmond, and Berkeley).

Due to the Covid-19 Pandemic, microsatellite analysis of samples collected during Year 1 was delayed. This was an additional complication to the already fraught process of sending fecal samples to Wyoming for extraction, PCR amplification at the Wyoming laboratory, sending microsatellite PCR products to Cornell in New York for fragment-size analysis, sending data files back to Wyoming for allele scoring, then sending analyzed allele scores to ROEP in California. In December, 2020, ROEP requested and received a one-year extension of our contract due to laboratory shut-downs and site closures during the pandemic. When the first 240 samples were eventually analyzed it was found that very few samples could be amplified for sufficient microsatellite loci for individual recognition, and some samples could not be amplified at all. A relatively low success rate from river otter fecal/jelly samples is expected, as such samples contain little DNA and many PCR inhibitors (Gallant et al. 2008), but success rates from these samples was even lower than originally expected. In June, 2022, ROEP requested and received permission to continue the study using a more recently-developed genetic analysis method to determine if better results were possible to overcome sample limitations.

For subsequent analysis we switched to use of single nucleotide polymorphism (SNP) methods, which can utilize high-throughput automated analysis and include much larger numbers of samples and loci analyzed quickly, and may also be more robust to low sample quantity and DNA degradation. Analysis of the same DNA extracts as used for the microsatellite analysis

would thus show whether the SNP approach is truly more robust, and might also provide a much larger number of independent genetic markers useful for distinguishing individuals for our genetic censusing analysis.

Materials and Methods

Study Area

ROEP has surveyed approximately 225 linear km of coastline, stream, estuary, and reservoirs in Marin County, California for active river otter latrines and movement corridors (Bouley et al., 2015; Carroll et al., 2020). Based on these surveys, eight geographically separated focal study sites (FSS) of different habitat types (Carroll et al. 2020) were selected to estimate abundance of river otters in Marin (Figure 2). We visited each FSS 1x/week during June-November when otters are active with young to check cameras and collect fecal samples.

Figure 2. Locations of the 8 focal study sites (FSS) where camera trap data and fecal samples were collected in 2019.

Camera Trapping

Motion-activated trail cameras (various models from Bushnell Outdoor Products, Overland Park, KS) recorded 10–60 second videos, 24 hours/day. Data extracted from each camera record included location, date and time of otter detections, number of adults and/or pup-juveniles per detection, and behavior including vocalizations. We estimated a) total, b) adult female, and c) pup abundance using methods from Bouley et al. (2015) at each FSS. A minimum abundance estimate is the largest grouping of river otters observed together on a single video from that location. For estimating density, we mapped the camera locations using ArcGIS (ESRI, Redlands, CA), and then measured the total linear shoreline length within a 7 km radius of the camera. The 7 km radius corresponds to the estimated home range size of river otters in coastal California (Brzeski et al., 2013; Bouley et al., 2015). Because river otters are rarely found more than 100m from watercourse and ocean shorelines (Bowyer et al., 2003), and approximate a linear pattern in their movements, shoreline length represents a better measure of otter distribution than area (Blundell et al., 2001). Briefly, a circle with a radius of 7 km was drawn around each FSS. For sites with multiple cameras, we determined the midpoint of the camera locations as the center of the circle. We then measured the length of coastline or watercourse in that area with the implicit assumption that all points of shoreline in the circle were equally likely to be used by the otters (Figure 3).

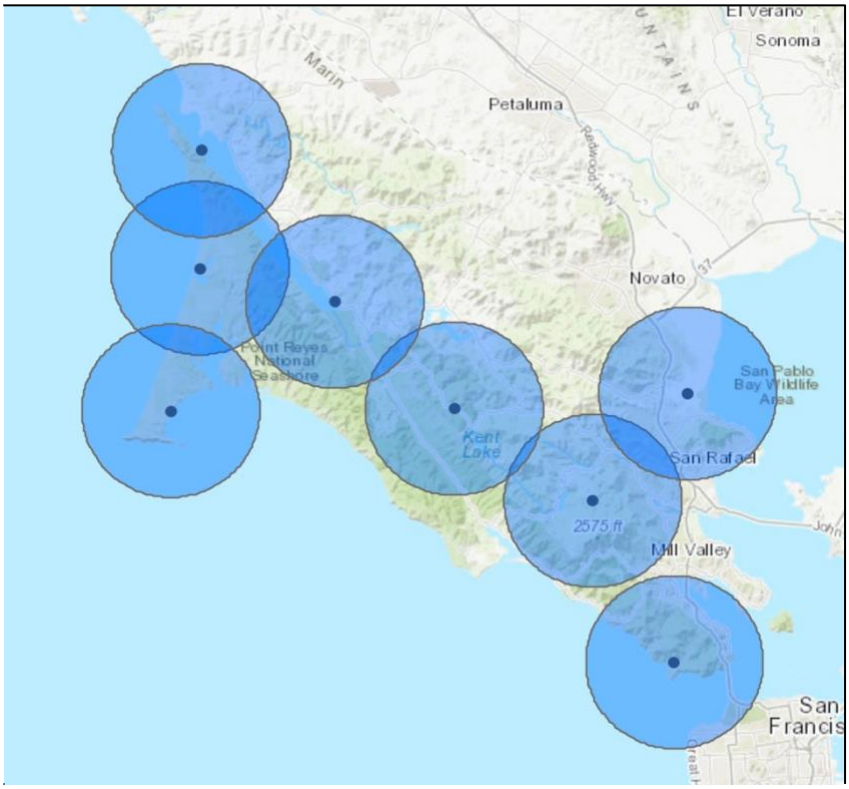

Figure 3. Map of River Otter Ecology Project's eight focal study sites (FSS) in Marin County, CA overlaid with home range estimates (blue circles). Total linear shoreline length was measured within each 7 km radius circle using ArcGIS (ESRI, Redlands, CA). The area of each circle is 154 km^2.

Fecal Sample Collection

Sampling was concentrated on known river otter FSS to maximize the probability of detection. Trained volunteers visited each FSS 1 - 2 days each week to collect fecal samples during July through September when it is assumed otters are neither emigrating nor immigrating (Harris and Ogan, 1997; Brzeski et al., 2013; Carroll et al., 2020).

Based on the protocol outlined for microsatellite analysis in Godwin et al. (2015), fresh scat and anal jelly (<24 h) was collected into individual 50 ml sample tubes, preserved in 95% ETOH, and stored at the Marin Academy Research Collaborative Laboratory frozen at -20°C.

Microsatellite Analysis

Extraction: Preserved fecal samples were transferred to the University of Wyoming for DNA extraction and amplification of microsatellite loci for a set of up to 12 microsatellite loci and 1 SRY locus by members of the Ben-David Laboratory. After sieving of samples through fine mesh stainless steel autoclavable sieves to remove prey item hard parts and homogenize the distribution of shed target cells, DNA was extracted from the scat samples using the QIAamp DNA Stool Mini Kit (QIAGEN, http://www1.qiagen.com). To monitor for contamination, a negative control (no DNA) was included with each batch of 10 sample extractions. There was not enough material for a second extraction after sieving.

Amplification: DNA was amplified by polymerase chain reaction (PCR) using a PTC-0200 DNA Engine Peltier Thermal Cycler (MJ Research, Inc., Waltham, Massachusetts, USA). Four microsatellite primers for PCR amplifications developed for Eurasian otters (*Lutra lutra*; LUT-701, LUT-733, LUT-801, and LUT-829; Dallas & Piertney 1998) were used, along with an SRY locus for genetic sexing. Five microsatellites developed for river otters (*Lontra canadensis*; RIO-01, RIO-02, RIO-05, RIO-17, RIO-19, RIO-20; Beheler et al. 2004, 2005, Mowry et al. 2011; (Table 1) were also used. Between 2 and 3 primers were amplified together in a multiplex based on the size of the product (i.e., number of base pairs) and fluorescent dye combinations (Table 2). Multiplexing cocktail information is included in Table 3. Thermal cycle protocols are listed in Table 4. Positive (blood samples from river otters with known genotypes) and negative (PCR blank) controls were included with each PCR reaction to ensure the reliability of PCRs and to monitor for contamination (Hansen et al. 2008). Resulting amplicons were diluted in formamide + LIZ500 fluorescent sizing standard and sent to the Cornell University Institute of Biotechnology Genomics Core Facility. There the amplicon fragment lengths were resolved on an ABI 3730xl Automated Sequencer (Applied Biosystems [ABI], Foster City, California, USA). The maximum number of amplifications per sample per primer varied between 3 and 7.

Table 1. Fluorescent dye and multiplexing options for 10 river otter primers and the sex determining gene SRY. Range in base pairs (bp) reflects published sizes. Number of alleles refers to samples included in this project based on consensus genotypes. Multiplex combinations are listed in Table 2.

Locus	Forward Labeled Primer	Reverse Unlabeled Primer	Multiplex	Number of Alleles	Range (bp)
Rio-01	PETAAGGGCACCTCGAGACAAT	CATGCTTGACCTTGAGCAAC	MP2	8	268-292
Rio-02	6FAMTAGAGTGGGGCGCCTAAGTT	TTACTCGCCAATGGTTCAGC	MP1, MP5, MP9	6	118-136
Rio-05	NEDGGGTTAAAGCCTCTGCCTTC	AGGGGATACCGGATCATTTC	MP3, MP6, MP9	5	310-354
Rio-17	6FAMGGTTGCGAAGATAAGCAAGG	CAAGTGTTTAAAGTGTGTGTGTGT	MP3, MP7, MP8	6	169-181
Rio-19	6FAMGGTCCCAGGTGCAAATCTTA	GATTTGGGTCTTCCAATGGTT	MP4, MP9	3	268-288
Rio-20	6FAMCTAGCTCTGCCACCTAACCAG	ACAGCGTGGTCCTGACCTT	MP5, MP6, MP7	4	242-262
Lut-701	NEDGGAAACTGTTAAAGGAGCTCACC	CAGTGTTCATAAGGATGCTCCTAC	MP1, MP6, MP8	4	190-210
Lut-733	6FAMGATCTCATTTTAAATGTTCTTACCAC	TGGTTCTCTTGCAGGATCTG	MP2	4	242-258
Lut-801	PETCATGATTAGAAGTTTGATTTGTGTTC	CATAATCACATGAGCCAATGC	MP1, MP5	2	226-238
Lut-829	PETCATGTCTGTCTTACACAGTCTATTCC	GAATTTGGATGGCATCAGGTC	MP3, MP8	6	224-256
SRY	PETGAATCCCCAAATGCAAAACTC	GGCTTCTGTAAGCATTTTCCAC	MP4, MP7	1	70

Table 2. Primers included in each multiplex for amplification of river otter DNA extracted from fecal samples.

Multiplex Number	Microsatellite primers
MP1	Rio-02 , Lut-701, Lut-801
MP2	Rio-01, Lut-733
MP3	Rio-05, Rio-17, Lut-829
MP4	Rio-19, Lut-829, SRY
MP5	Rio-02, Rio-20, Lut-801
MP6	Rio-05, Rio-20, Lut-701
MP7	Rio-17, Rio-20, SRY
MP8	Rio-17, Lut-701, Lut-829
MP9	Rio-02, Rio-05, Rio-19

Table 3. Multiplexing cocktail for amplification of river otter DNA extracted from fecal samples.

PCR reaction mixture	Amount (µl)
Sample	1
ddH_2O	1.8 (generally, depending on number of primers included in the multiplex)
Qiagen Multiple PCR Plus Master Mix	4
Forward labeled primer	0.2
Reverse primer	0.2
Total volume (µl)	8

Table 4. Thermal cycler protocols for amplifying of river otter DNA extracted from fecal samples. Group 1 includes: Rio-02, Rio-05, Rio-17, Rio-19, Rio-20, Lut-701, Lut-801, Lut-829. Group 2 includes: Rio-01, Lut-733.

PCR reaction step	*Group 1*	*Group 2*
1. Activation	95° for 5:00	95° for 5:00
2. Denaturation	95° for 0:30	95° for 0:30
3. Annealing	63° for 1:30	51° for 1:30
4. Extension	72° for :30	72° for :30
5. Cycles	Goto 2, 35 times	Goto 2, 35 times
6. Final extension	68° for 30:00	68° for 30:00
7. End of cycling	4° forever	4° forever

Scoring and Genotype Calling: At the Wyoming laboratory, resulting assay files for microsatellite products were scored using the package Fragman in R (R computing 2020). Amplification of all samples was attempted twice with an initial set of 8 microsatellite loci. After analyzing those 8 loci, samples that did not yield reliable results for at least 2 of the loci were omitted from further analysis. Samples that amplified as a heterozygote 2X or homozygote 3X were considered correctly called. If an allele occurred multiple times but with an inconsistent partner, that allele was included in the consensus genotype, and only that allele is listed. If a sample amplified homozygote 2X after up to 7 reactions, it was included in the consensus genotype and marked in Italics. Genotyping data are provided in the Supplementary Materials file "ROEP_Genotypes.xlsx".

The Ben-David lab conducted the fragment size analysis from the resulting .fsa files which were then provided to ROEP; analysis of genotype profiles for individual identification, estimation of number of individuals, home ranges and boundaries for each putative population were analyzed by Frank Cipriano and Terence Carroll.

SNP Analysis

KASP Allele-specific Hybridization: Methods for discriminating between alleles at a SNP locus are typically based on hybridization using allele-specific fluorescently-labelled oligonucleotide probes to produce a fluorescent signal indicating one or the other homozygote or 'mixed' heterozygotic sequences detected during (real-time PCR detection) or after (end-point detection) thermal cycling (various methods are reviewed in Chopra 2014). Two probes are required, one specific for each allele, and stringency conditions are employed such that a single-base mismatch at the 3' end is sufficient to prevent hybridization of the non-matching probe. The KASP assay uses a unique form of allele specific PCR developed at LGC Genomics (http://www.lgcgenomics.com/).

SNP Genotyping Panel: Ninety-six SNP markers for river otters were developed by Stetz et al. (2016), from over 20,000 loci identified using restriction-site associated DNA (RAD) sequencing. After careful filtering to minimize ascertainment bias and identify high quality, highly-

heterozygous SNPs, they designed a panel of 52 independent SNP-genotyping assays. Testing showed that 41 of those loci performed well even with diluted DNA, and provided high power for population assignment tests. We used the set of 52 loci to genotype almost all of the same river otter samples as attempted for the microsatellite study, using LGC-KASP chemistry (KBioscience Ltd., Hoddesdon, UK) on Fluidigm Nanofluidic 96.96 Dynamic Arrays (Wang et al., 2009) which run 96 samples against 96 assays (9,216 reactions at once), which can be cycled and then analyzed in just a few hours. It should be noted that the minimum amount of each assay and accompanying pre-amplification primers that we ordered is sufficient to run many thousands more samples; even with the additional cost of pre-amplification reagents, Fluidigm IFC 'chips' and Master Mix reagents for on-chip reactions, the per sample cost of additional sample analysis will thus be reduced compared to this initial study.

Assay Protocol: For genotyping using KASP chemistry, sample mix and assay mix were prepared according the manufacture's protocols, as modified by Thaden et al. (2020) for low quality/quantity DNA extractions. Each sample was first 'pre-amplified' using primers also generated by LGC for the Stetz et al. loci, with 3µl of DNA extract in 8 µl total reaction volumes using a Qiagen multiplex reaction kit and with the cycle count increased to 25 cycles. The assay mix and pre-amplified sample mix were then loaded onto a 96.96 dynamic array chip using a Fluidigm HX integrated fluidic circuit (IFC) Controller, amplified using an FC1 thermal cycler, and end point reactions then quantified with an EP1 fluorescence reader (Standard BioTools., South San Francisco, CA) with reaction volumes and cycling conditions according to the manufacturer's protocols. Since the 96:96 chips can hold up to 96 different assays, we repeated the use of 44 assays and ran 93 samples per run, with three no template controls reserved on each chip, for a total of 186 samples. The resulting data was analyzed using the Fluidigm SNP Genotyping Analysis Software. Dye settings used for data analysis were MGB-VIC and MGB-FAM.

Error Checking and Match Analysis: After checking for mismatches and consolidation of replicates into a single set of calls for each sample/locus combination, and removing unsuccessful samples and loci, we used MicroSatellite Toolkit (an Excel add-in, Park 2001) to calculate basic statistics and compare samples to identify matches.

Results

Camera Trapping

A total of 1,444 videos were indexed, compiled, and analyzed to estimate a) total, b) adult female, and c) pup abundance using methods from Bouley et al. (2015). Monthly camera trap abundance estimates also were determined for each FSS for future comparison with our genetic data abundance estimates.

Total minimum abundance estimates ranged from 3 to 7 otters with the fewest number of otters recorded at Rodeo Lagoon and the greatest number recorded at the Tomales Bay sites (Table 1).

The fewest number of otters (n = 21) were recorded during October, 2019 and the greatest number of river otters (n = 42) were recorded during July, 2019. Overall, we estimated a minimum abundance of 45 otters across all sites. Adult females with pups only were observed at three locations including Northern Tomales, Abbotts Lagoon, and Las Gallinas (Table 5).

River otter density ranged from 0.06 to 0.19 otters/km (Table 6). Densities were highest at the Northern and Southern Tomales Bay, and Abbotts Lagoon FSS, and lowest at Rodeo Lagoon.

Table 5. Camera trap data collected during Year 1 including focal study sites, number of otters estimated for each month, and overall numbers of otters, adult female otters, and pups.

Focal Study Site	June	July	August	September	October	All Months	Adult Female	Pup
Northern Tomales Bay	4	7	7	7	7	7	1	3
Abbotts Lagoon	3	6	4	4	4	6	1	2
Southern Tomales Bay	1	7	1	7	5	7	nd	0
Drakes Bay	5	6	6	6	4	6	nd	0
Las Gallinas	5	5	--	--	--	5	2	3
Middle Lagunitas Creek	1	6	6	4	2	6	nd	0
Reservoirs	2	3	3	5	3	5	nd	0
Rodeo Lagoon	2	2	2	1	3	3	nd	0
Totals	**23**	**42**	**29**	**34**	**21**	**45**	**3**	**8**

-- indicates no data collected
nd indicates the number of females could not be determined from camera data

Table 6. Camera trap data collected during Year 1 including focal study sites, linear shoreline length, overall river otter estimate, and density.

Focal Study Site	Linear Shoreline Length (KM)	Number of Otters	Density/KM
Northern Tomales Bay	58.70	7	0.12
Abbotts Lagoon	39.66	6	0.15
Southern Tomales Bay	36.45	7	0.19
Drakes Bay	52.99	6	0.11
Las Gallinas	33.67	5	0.15
Middle Lagunitas Creek	53.37	6	0.11
Reservoirs	37.17	5	0.13
Rodeo Lagoon	49.59	3	0.06

Fecal Sample Collection

We collected a total of 347 samples from the 8 FSS. Of that total, we sent 240 samples to the Ben-David lab at University of Wyoming for microsatellite analysis (Table 7).

Table 7. Number of fecal samples collected at each focal study site, and the number that were sent for microsatellite analysis.

Focal Study Site	Samples Collected	Samples Sent for Analysis
Northern Tomales Bay	66	61
Abbotts Lagoon	65	65
Southern Tomales Bay	31	30
Drakes Bay	29	28
Las Gallinas	37	18
Middle Lagunitas Creek	39	6
Reservoirs	57	12
Rodeo Lagoon	23	20
Totals	**347**	**240**

Microsatellite Analysis

From the 240 samples tested in the microsatellite genotype analysis, a large number of samples failed to amplify or the amplifications were so weak they could not be scored. A genotype sufficient for distinguishing among samples was obtained for 28 individual otters, from samples collected at 6 of the 8 FSS. In 3 instances, samples from among these individuals were collected twice, or 'recaptured'. The number of individuals identified ranged from 1 at Southern Tomales Bay to 10 at the Abbotts Lagoon. No individuals were identifiable from samples collected at Drakes Bay or from the small number of samples from Middle Lagunitas Creek sent for analysis.

SNP Analysis

As with the microsatellite analysis (performed on 186 of the same DNA extracts from the same samples), a large number of samples almost totally failed to amplify (n=132) or amplified for only a small number of loci out of the 52 tested. From the 186 samples tested in the SNP genotype analyses, a genotype sufficient for identification was obtained for 27 individual otters, from samples collected at 3 of the 8 FSS.

Fluidigm SNP analysis of river otter samples with the set of 52 Stetz et al. 2016 assays produced results we divided into three categories according to the number of samples amplified and whether informative or not (Table 8, Appendix 1).

Table 8. Categories used to distinguish varying degrees of success with use of SNP loci.

Category	Number	Characteristics
category green – ok to use	11 of 52 loci tested	3 distinct clusters
category yellow – use with caution	12 of 52 loci tested	2 or 3 clusters, some smearing
category gray –uninformative	29 of 52 loci tested	only one cluster

More than half of the loci tested were in 'category gray', and appeared to have one or the other homozygote invariant ('fixed'). The remaining 23 loci appeared to be polymorphic, although some were difficult to score with the low number of samples that amplified for any of the loci we tested. Results from samples run on two different Fluidigm 'chips' showed similar patterns for each assay (Appendix 1), and among replicates run on the same chip, mismatches were scored in only 11 instances. Mismatches were checked and calls corrected from visual inspection of the fluorescence plots (Appendix 2).

We used MicroSatellite Toolkit (Park 2001) to calculate basic statistics (Table 9) for the SNP loci in categories green (n=11) and green plus yellow (n=23). The SNP loci selected for assays by Stetz et al. (2016) were developed using representative samples from across North America, with candidate loci filtered to obtain highly heterozygous assays (Ho = 0.2–0.50) SNPs and minimize ascertainment bias. Although based on relatively few successful genotypes, the magnitudes of the observed and unbiased heterozygosities of the loci in the two 'successful' categories we analyzed appeared similar, and within the desired informative range.

Table 9. Unbiased and observed heterozygosity (std. deviation) and number of alleles diagnosed for two sets of SNP loci (23 'all' and 11 'best').

Sample size	Loci typed	Unbiased Hz (SD)	Obs Hz (SD)	No. Alleles
64	11	0.441 (0.021)	0.265 (0.024)	2
64	23	0.385 (0.027)	0.300 (0.018)	2

We also used MicroSatellite Toolkit (Park 2001) to compare samples to identify matches among 64 sample pairs, using both the best assays (11 in category green) and all informative assays (23 in categories green and yellow). Analysis of both the category green, and category green plus yellow genotypes each found 6 instances where samples from among these individuals were obtained twice, or 'recaptured'. The number of individuals identified and recaptured included 1 at Rodeo Lagoon, 1 at southern Tomales Bay, and 4 at Abbott's Lagoon.

Comparing the Individual Identification and Matching Analyses

Results from the same samples analyzed using both microsatellite and SNP analysis were strikingly similar, in that only a small subset of the samples analyzed produced robust results for both methods (Table 10). In most cases, the 40 most successful samples analyzed using SNPs, almost all (green shading) also produced the best results in the microsatellite analysis. Four of the samples quite successful in the SNP analysis were less successful in the microsatellite analysis (sample IDs 190, 196, 100, and 97, Table 10). Another sample that performed poorly in the SNP analysis was more successful in the microsatellite analysis (sample ID 92, Table 10).

Table 10. Comparison of results from the 40 most successful samples for both microsatellite and SNP genotyping, ranked by SNP loci scored. Samples shaded in green were considered successful.

Sample_ID	Site	Date Collected (D/M/YY)	SNP Loci scored (Out of 96)	SNP Loci Scored (Out of first 52)	Msat primers amplified (Out of 11 loci)	Msat primers with consensus genotype
99	S Tomales	8/30/19	94	51	10	10
157	Abbotts	9/16/19	94	51	10	10
158	Abbotts	9/16/19	94	51	10	10
190	S Tomales	9/22/19	93	51	3	0
195	Abbotts	9/23/19	93	50	10	9
5	Abbotts	7/11/19	92	50	10	9
35	Abbotts	8/6/19	92	50	10	10
75	Abbotts	8/20/19	92	50	10	9
123	Reservoirs	9/5/19	92	50	10	10
153	Abbotts	9/13/19	92	50	10	8
192	N Tomales	9/22/19	92	50	10	10
196	Abbotts	9/23/19	92	50	3	1
119	Las Gallinas	9/4/19	91	48	10	10
194	Abbotts	9/23/19	91	49	10	10
32	Rodeo Lagoon	8/6/19	90	48	10	10
170	Rodeo Lagoon	9/19/19	90	49	10	10
23	N Tomales	7/25/19	86	46	9	9
178	N Tomales	9/21/19	85	46	10	9
109	Reservoirs	9/2/19	72	38	10	8
100	S Tomales	8/30/19	70	38	6	1
9	Rodeo Lagoon	7/19/19	66	36	10	8
98	S Tomales	8/30/19	64	35	9	0
97	S Tomales	8/29/19	60	32	5	0
18	N Tomales	7/23/19	59	31	10	6
180	N Tomales	9/21/19	56	30	10	9
14	Las Gallinas	7/20/19	48	26	10	8
149	Reservoirs	9/12/19	45	26	10	9
198	Abbotts	9/23/19	42	23	4	1
229	Abbotts	7/30/19	42	22	3	1
226	Abbotts	7/16/19	38	19	3	0
92	N Tomales	8/27/19	33	17	9	0
247			33	18		
95	Las Gallinas	8/29/19	32	17	5	0
156	Las Gallinas	9/14/19	31	16	6	3
219	Abbotts	7/11/19	29	17	4	1
20	Drakes Bay	7/23/19	26	14	3	0
26	Abbotts	7/30/19	24	14	4	0
91	N Tomales	8/27/19	23	11	4	0
118	Las Gallinas	9/4/19	22	12	6	0
33	Rodeo Lagoon	8/6/19	21	12	6	3

To estimate the number of individuals identified using the two methods we compared results from the microsatellite and SNP analyses based on the number of near-complete genotypes obtained using each method (Table 11). We added up the number of near-complete genotypes as an estimate of 'identifiable' samples, and then subtracted the number of plausible matched samples to estimate the total number of distinct individuals detected using each method. Results from both methods were similar, and again were essentially limited to the same apparently higher-quality samples tested using both methods.

Table 11. Samples identifiable using microsatellite and SNP analyses, and matched samples (e.g. 157/158) identified using both methods. Box around samples 194/18 indicates a cross-site match, demonstrating the potential for detecting movement between sites.

Focal Study Sites	Sample ID	Identifiable Msat Genotype	Identifiable SNP Genotype	Sample ID
Abbotts Lagoon	5	X	X	5
	34	X		
	35	X	X	35
	48	X		
	75	X	X	75
	81	X		
	152	X		
	153	X		
	157/158	XX	XXX	153/157/158
	195	X	XX	195/196
	194	X	X	194/
Northern Tomales	18	X	X	18
	23	X	X	23
	126	X		
	178/192	XX	XX	178/192
	179	X		
	180	X	X	180
Las Gallinas	12	X		
	14	X	X	14
	119	X	X	119
Reservoirs	109	X	X	109
	123	X	X	123
	124	X		
	149	X	X	149
Rodeo Lagoon	9	X	X	9
	32/170	XX	XX	32/170
	122	X		
Southern Tomales			X	97
			X	98
	99	X	XX	99/190
			X	100
IDable Sample/Indivs	28 indivs	31 samples	27 samples	20 indivs

From the analysis of the 11 'best' assays only analysis we note one 'recapture' that indicates the potential use of such analyses to track movement between adjacent sites, from samples obtained two months apart (in July and September 2019) at Abbot's Lagoon and northern

Tomales Bay. Although one of the samples had a relatively low call rate of 61.4%, all alleles that were called in common matched exactly.

Discussion

Specific Aims

This community science driven study accomplished the first aim of the proposed work: to estimate minimum population abundance and distribution of river otters at select sites in the SFBA using noninvasive camera-trapping techniques. Although constrained by fecal sample quality issues, we partially accomplished the second aim: to use genetic analyses to evaluate population differentiation of river otters in the SFBA and genetically identify individuals, then use capture-recapture analyses for abundance estimation. We also partially accomplished the third aim: to compare minimum population abundance estimates derived from camera-trapping and genetic analyses.

Due to the limited success with sending out samples for microsatellite analysis, exacerbated by complications from the Covid-19 pandemic, we altered the study design after Year 1 to include a new type of genetic analysis and a new specific aim: testing whether SNP analysis was more robust to poor sample quality, faster and more resource-efficient, and potentially less costly per sample. Although sample quality remains a critical limitation, the SNP analysis was a notably faster and more efficient process than the microsatellite analysis. Once the initial fecal sample DNA has been extracted, and the custom assays purchased, the marginal per-sample costs can be much lower and the samples can be processed relatively quickly using high-throughput techniques if a laboratory with suitable equipment is available.

Pending future analyses with higher-quality samples, we have not yet accomplished the fourth original aim: to determine whether a consistent correction factor can be used across sites and years to make accurate abundance estimates from camera trap data. With sufficient comparative data accumulated, it will also be possible to evaluate whether statistically adjusted camera trapping abundance estimates can be as accurate as those from genetic methods, under what circumstances one can substitute for the other, and which is ultimately more cost-effective.

Camera Trapping

Results from the camera trapping portion of this study were broadly consistent with prior FSS abundance estimates from our long-term monitoring program (Carroll et al., 2020). Our estimates of minimum site abundance assume closed populations during the months of June through October at each of the study sites (Brzeski et al., 2013, Bouley et al., 2015; Carroll et al., 2020). Given the geographic separation of our sites, and based on an earlier study in Humboldt County, we assumed that individual otters travel infrequently, if at all, among sites during the months when the minimum abundance estimate for each site was determined (Brzeski et al., 2013). We also assumed that any such travel was episodic rather than representing recruitment from one site to another, minimizing the likelihood of counting the same otter at more than one site.

The spatial pattern of differences in estimates of river otter density among the various FSS is consistent with the analysis of our long-term monitoring results reported by Carroll et al. (2020) in that more northerly sites show greater density of river otters than more southerly sites. Overall, however, our estimates of river otter density at each FSS are low compared to previous studies of coastal river otters (Testa et al., 1994; Ott, 2009; Brzeski et al., 2013; Bouley et al., 2015). Our assumption of equal probability of use of all shoreline points within a 7 km radius of a study site may not accurately represent actual otter use of home range areas (Blundell et al., 2001). If otter use of home range area is mainly confined to a core area of shoreline within the home range, our methodology would overstate shoreline length, resulting in lower density estimates. In addition, the objective of our study was to estimate density at a site level, rather than at a study area level as in earlier studies, so our results may not be directly comparable to those of the earlier studies. Our methodology, however, by assigning an equivalent area to each site, allows for direct comparison of density estimates among the sites, which is relevant for our objective of informing the planning for, and response to, toxic spills.

Fecal Sample Collection

Results from both the microsatellite and SNP analysis were severely limited, likely due to low quality and low quantity DNA isolated from otter fecal samples. Defects in our fecal sample collection methodology likely contributed to the poor quality of results from our genetic analysis. In particular, we found that the amount of fecal matter we collected in each sample was in many cases inadequate for DNA extraction. In addition, shipping of samples to Wyoming likely contributed to degradation of sample quality. For future studies, we recommend that larger amounts of fecal matter be collected for each sample, and that DNA extraction be done locally to minimize degradation from shipping and handling.

Microsatellite Analysis and SNP Analysis

Sending out our samples for extraction, microsatellite amplification and fragment size analysis resulted in a total of 31 samples and 28 individuals successfully genotyped, with 3 recaptures identified, out of the 240 samples attempted (Table 10). This 13% success rate is low compared to previous studies. For example, Mowry et al. (2011) reported a success rate of 24%, and Brzeski et al. (2013) reported a 25% success rate. As noted above, the main factor contributing to our lower success rate was likely an insufficient amount of fecal matter collected in each sample, which also did not allow for repeated attempts at DNA extraction from such low-quality samples.

The 52 Fluidigm assays were used to test 186 of the 240 extracted river otter DNA samples used for the microsatellite analysis. Twenty-seven samples were identifiable from genotypes obtained from the higher-quality DNA extracts, for almost the full set of 23 informative loci. Deducting the recaptures, which were almost exclusively intra-site, produced an estimated 20 individuals identified using the SNP analysis. One lower-stringency recapture ('boxed' sample id's 194/18 in Table 11) is plausible evidence for movement of an individual between adjacent sites, given the distance between the two locations and sampling dates about two months apart. SNP replicates (samples run twice for 44 of the 52 assays) produced similar results, with

only 11 instances where different alleles were called using automated and manual inspection of position in the fluorescence plots. In most cases, these differences resulted from a 'no call' scoring of one replicate, with an actual allele called for the other. Visual checking of these results allowed allele assignment in these cases with little apparent ambiguity (Appendix 2).

Cost and Efficiency

Although SNP amplification is generally expected to be more robust than microsatellites because smaller DNA fragments are amplified, our results for individual samples using both techniques were strikingly similar – and only the 'best' of our relatively low-quality DNA extracts derived from fecal samples could be successfully genotyped using either approach (Table 10). Yet even with results from a relatively small number of the SNP loci it was possible to start identifying recaptures of individuals at the same sites on different days, and distinguishing between individuals within a site on the same day. Further, the entire process for the SNP analysis of 186 of the original 240 samples using two 96:96 Fluidigm chips was completed in three days using DNA extracts left over from the microsatellite assays. In contrast, the microsatellite analysis of the original samples took a number of months, and involved two different labs for DNA extraction, amplification, shipment of products, fragment sizing, exchange of data files between labs, allele filtering and calling, and a final matching process. Even discounting the Covid-related delays and complications in our microsatellite analysis, we found clear cost and efficiency advantages to the SNP approach of running 96 samples against 96 assays in a matter of days. The DNA extraction step remains the largest limiting factor in the SNP approach.

Results from our first attempt at SNP analysis appear to have been limited due to fecal sample and resulting DNA extract quality issues. In addition, many of the SNP assays from the Stetz et al. 2016 SNP panel we tested appear to be monomorphic in samples from our study area, albeit with relatively low sample sizes given the number of failed amplifications. Some of those assays might still be informative with respect to samples from other geographic populations. In addition, some 'missing' alleles might be discovered given more fecal samples with sufficient material for effective DNA extraction. The remaining 44 assays from the complete Stetz et al. SNP panel would likely include some that could also be amplified and be informative within our local population, especially if positive controls from tissue extracts of known-source individuals were included throughout the process to help identify and correct handling and protocol errors. Our ability to score alleles will also improve as more samples are added and assayed, because the pattern for each locus analyzed is somewhat unique and with larger sample sizes such patterns become clearer. We now have direct experience with setting up pre-amplification reactions, and preparing materials for the Fluidigm assay, provide a solid foundation for future studies using the SNP approach.

Comparison of Camera Trap and Genetic Analysis Population Abundance Estimates

Our study did not produce sufficient genetic data within and across sites to allow us to meaningfully model the relationship between camera trap abundance estimates and estimates based on genetic analysis of fecal samples. Even with poor quality fecal samples, however, we did succeed, with both microsatellite and SNP analysis, in identifying individual genotypes and

recaptured individuals within multiple sites, thus validating our general approach and methodology.

Conclusions and Suggestions for Further Study

San Francisco Bay is home to some of the busiest ports on the west coast; the Port of Oakland alone is the seventh busiest port (by cargo volume) in the United States. Bay Area river otters are at risk from spills in this high-traffic area, and the need for information on their population abundance and distribution is clear. Our study compared two commonly-used techniques for abundance and distribution estimation, to determine whether camera trapping yields results comparable to those gained through DNA identification and capture recapture analysis.

Our team also began exploring the use of Single Nucleotide Polymorphism (SNP) technology to reanalyze samples collected in the first field season and submitted for microsatellite analysis, for comparison of results so that we can complete objective 2) Use genetic analyses to evaluate population differentiation of river otters in the SFBA and genetically identify individuals, then use capture-recapture analyses for abundance estimation and 3) Compare minimum population abundance estimates derived from camera trapping and genetic analyses. Our belief is that SNP technology may produce more successful results than microsatellites for the samples we collected. If so, using SNP technology will provide a better overall outcome and enable us to determine whether a consistent correction factor can be used for data collected from a different set of sites.

With such improvements, use of SNP analysis could allow a range of analyses of vital importance including insight into the demography and distribution of wildlife species in California to monitor survival of affected wildlife after spills. Reliable individual identification would allow tracking movements of individuals between sites, providing critical data on dispersal patterns and the possibility of changes in occupancy patterns post-spill. In addition, determining kinship relationships could provide information on localized changes in genetic diversity post-spill.

Development of new SNP assays by leveraging information from the loci we tested against chromosome-level genome sequences could add loci effective within and between a wider range of river otter populations, and help develop similar tools for use with other otter populations and species.

We initially assumed that camera trapping would be significantly easier and potentially more cost-effective than genetic methods, and thus could be used more broadly by volunteer community scientists directed by professionals. Our results suggest, however, that over the longer-term SNP analysis might be a more cost-effective and informative monitoring tool. The kit-based DNA extraction method used for fecal samples is relatively expensive, but requires minimal investment in additional equipment. Training in the procedure could allow a dedicated subset of volunteers to extract the DNA locally as new samples are accumulated. The extracted DNA is suspended in buffer and is more stable over time than frozen fecal samples.

Both methods require an initial investment in equipment and training time, but subsequently the marginal cost of additional sampling using SNP analysis may be less than that for camera trapping. Moreover, in contrast to camera trapping, SNP analysis can identify individuals and kinship relationships, and so each sample may be more informative per unit cost. Future studies could explore the relative costs and benefits in this context.

Significance to oiled wildlife health

Both noninvasive camera and genetic techniques are promising approaches for generating qualitative and quantitative information on distribution and abundance of river otters, including information about pup emergence time and survival. Integrated abundance estimates from both techniques likely will provide a more repeatable and reliable assessment of otters in the San Francisco Bay Area, their vulnerability to spills, and the potential investment of resources for oil spill response. We demonstrated the viability of our approach in the context of a community science effort through which members of the public can be engaged to participate in relatively technical research programs related to toxic spill preparedness.

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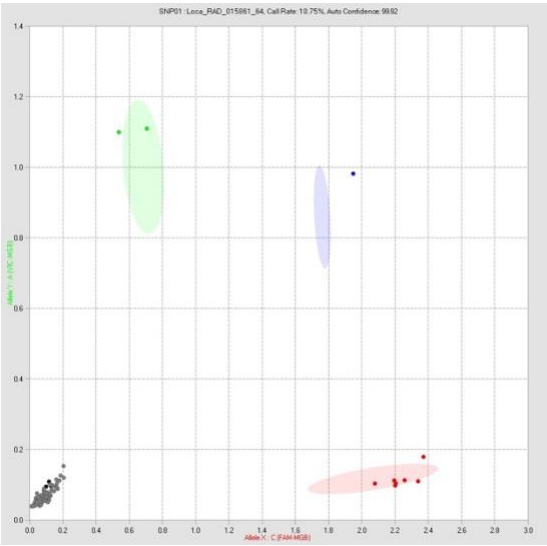
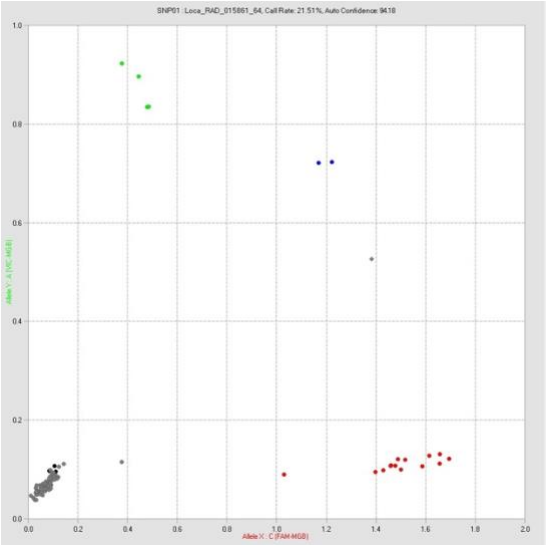
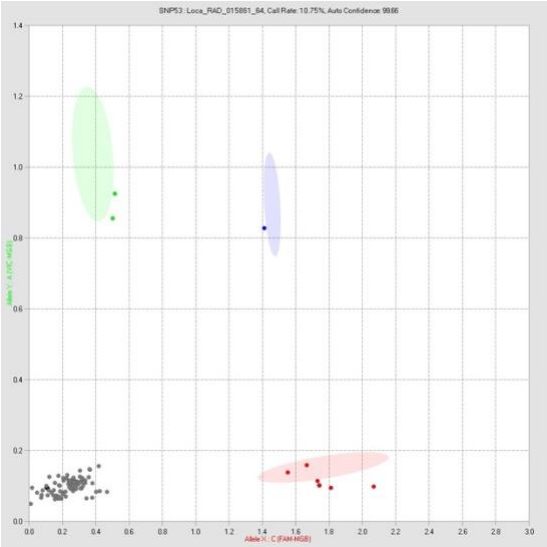
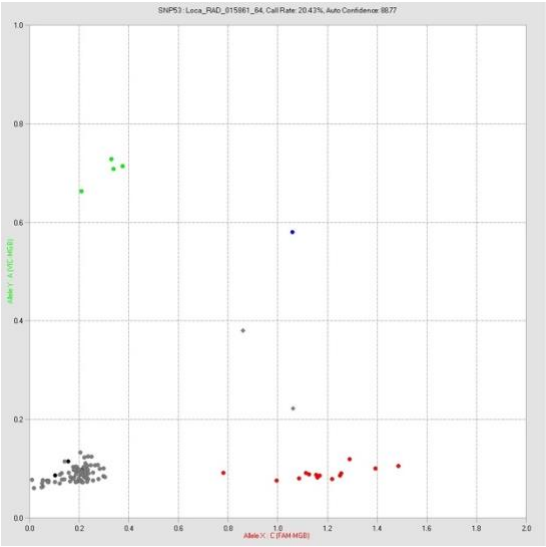
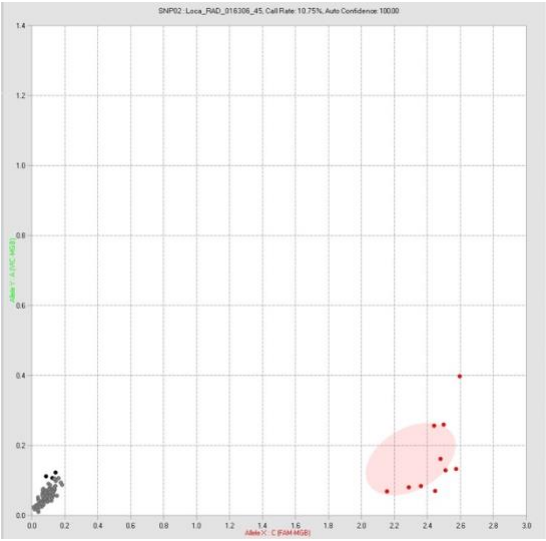
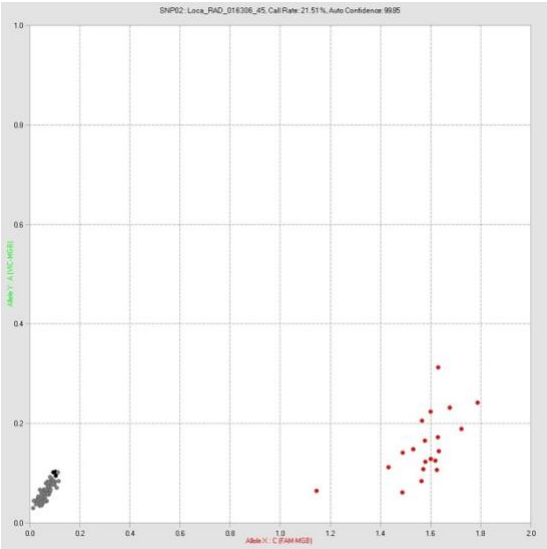
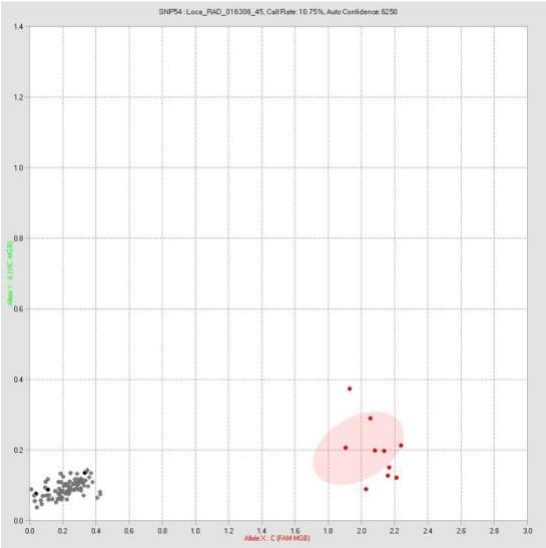
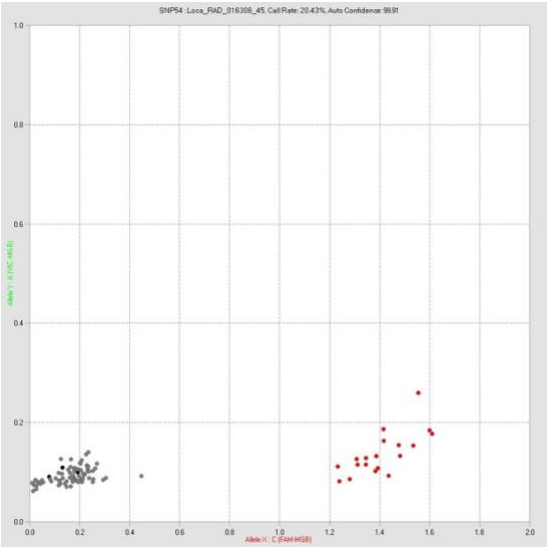
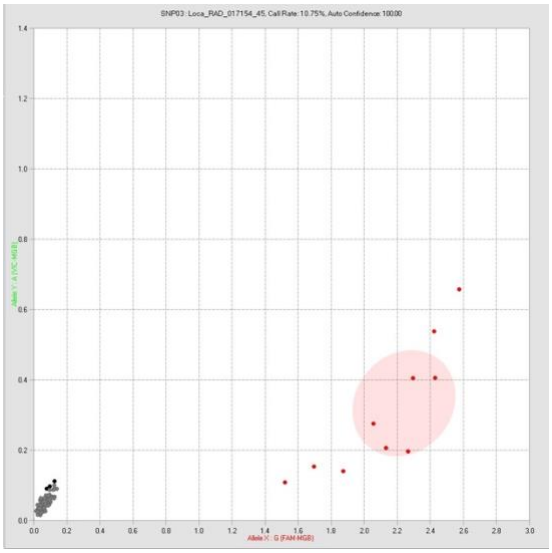
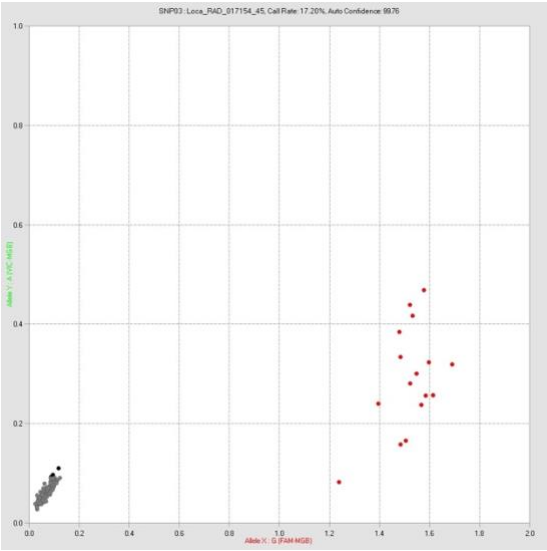
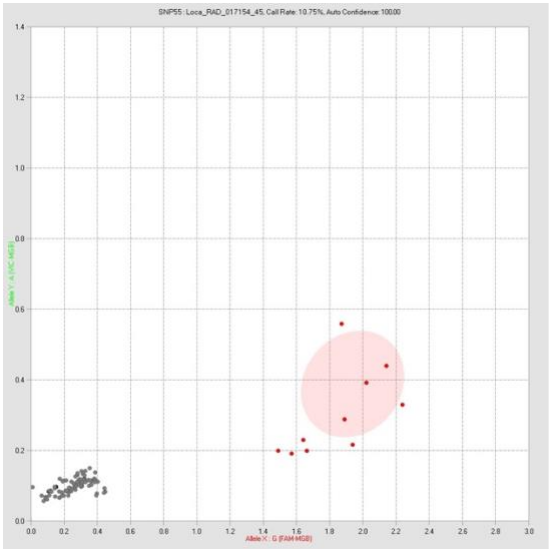
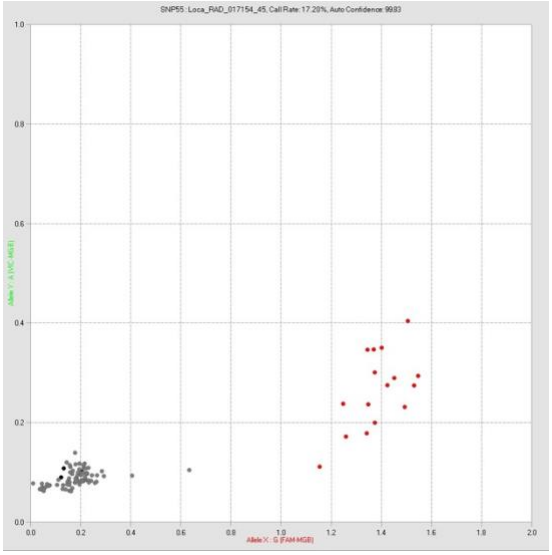
Appendix 1. Fluorescence plots for all 52 SNP assays plus 44 repeated-assay replicates on two Fluidigm 96:96 dynamic array integrated fluidic circuit (IFC) 'chips'. Colored ovals in Chip 1 (left hand panel) results represent approximate spread of results from Chip 2, as an aid to help with discriminating allele calls. Notes at the bottom summarize observations and the success category assigned to each assay.

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP01	Loca_RAD_015861_64

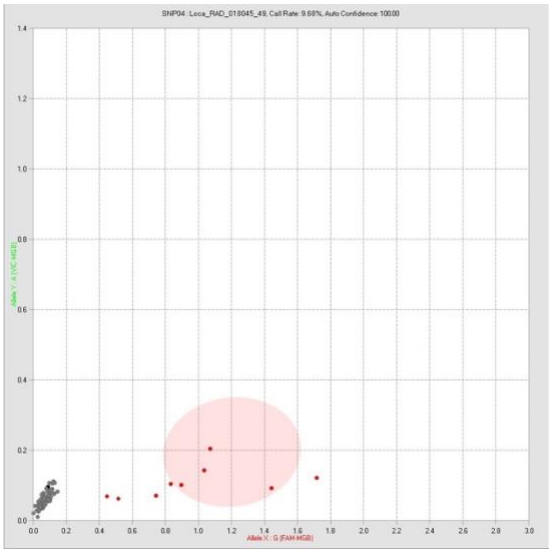
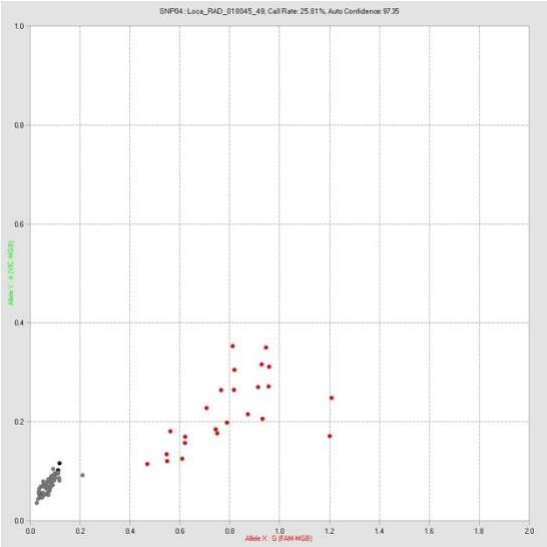
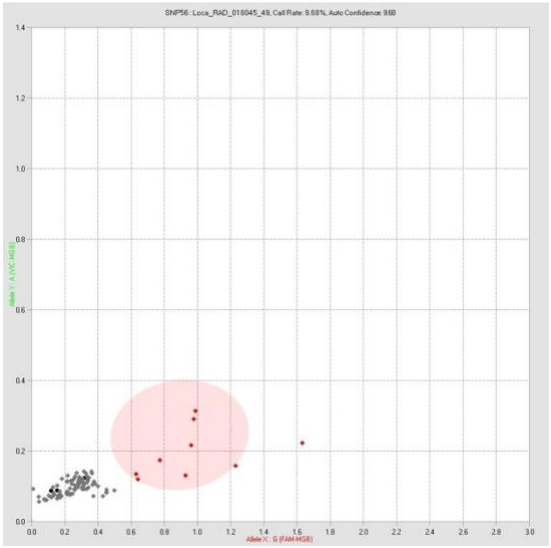
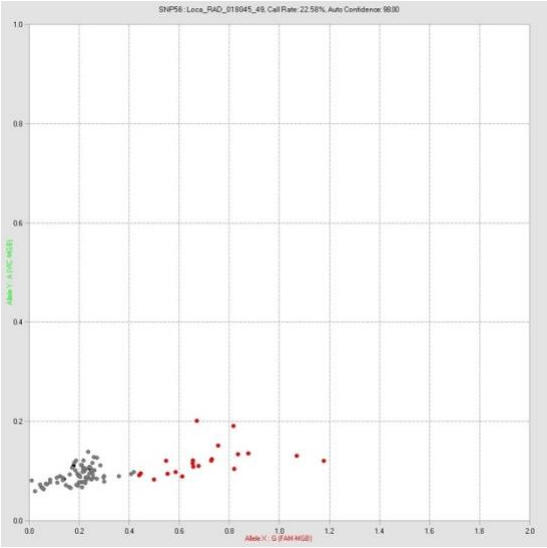
some samples fail, but 3 distinct groupings
category green (ok to use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP02	Loca_RAD_016306_45

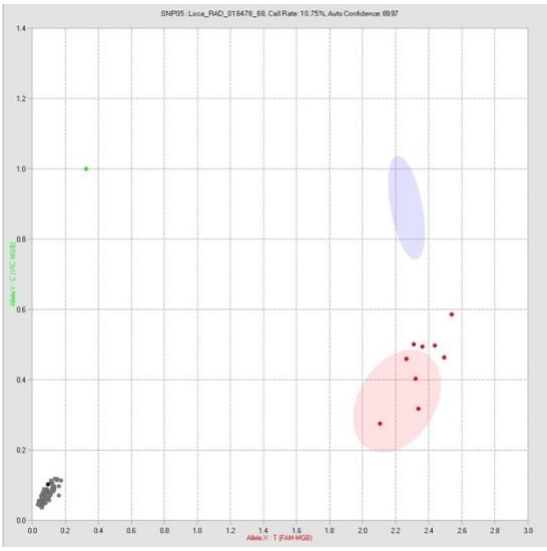
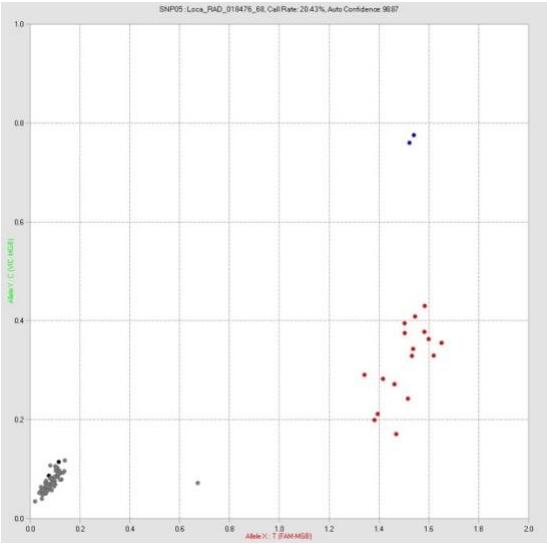
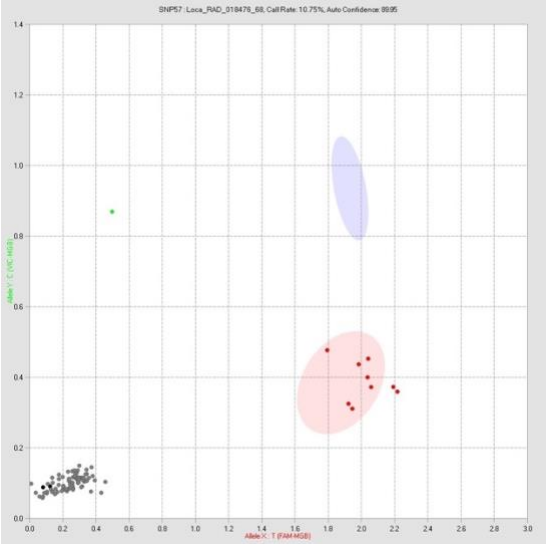
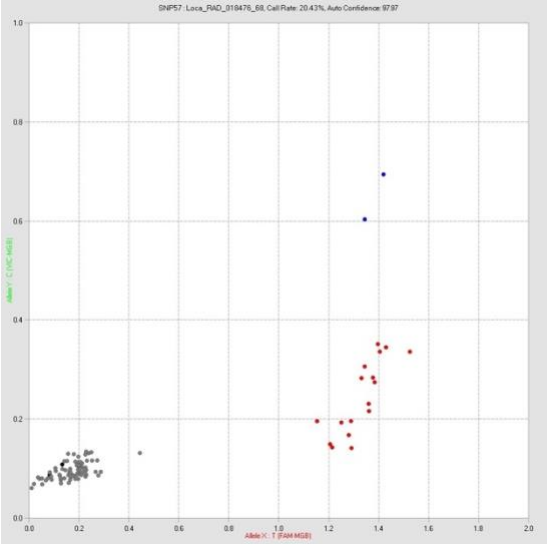
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP03	Loca_RAD_017154_45

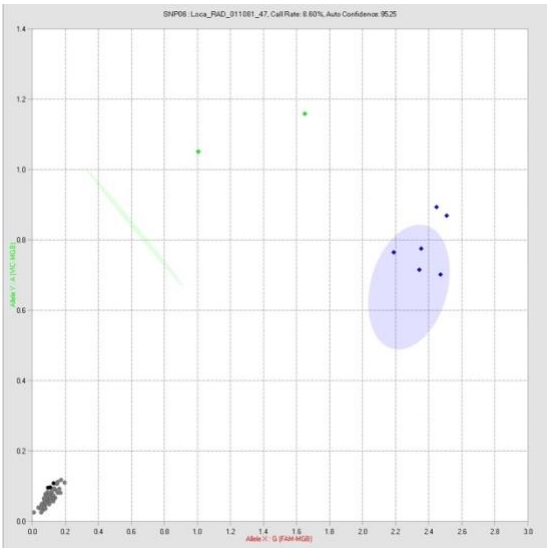
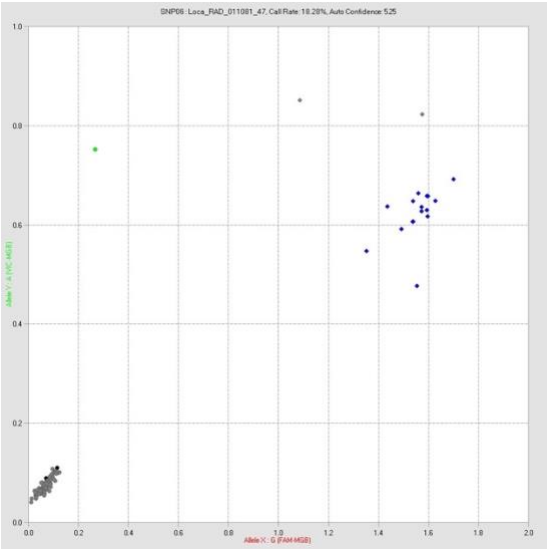
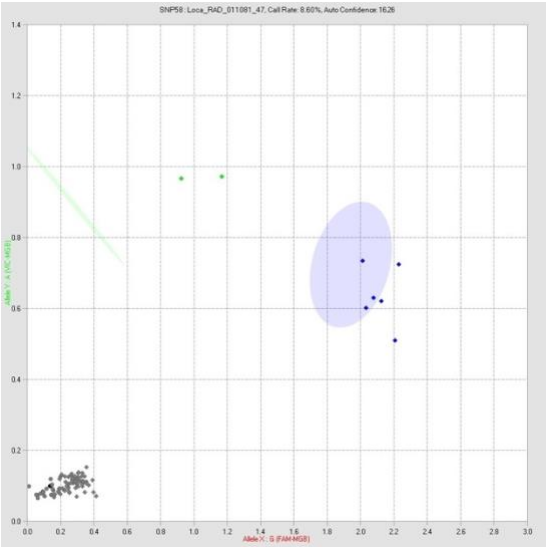
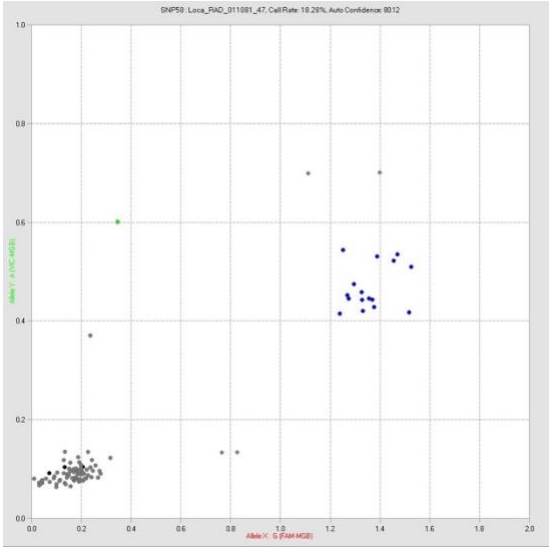
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP04	Loca_RAD_018045_49

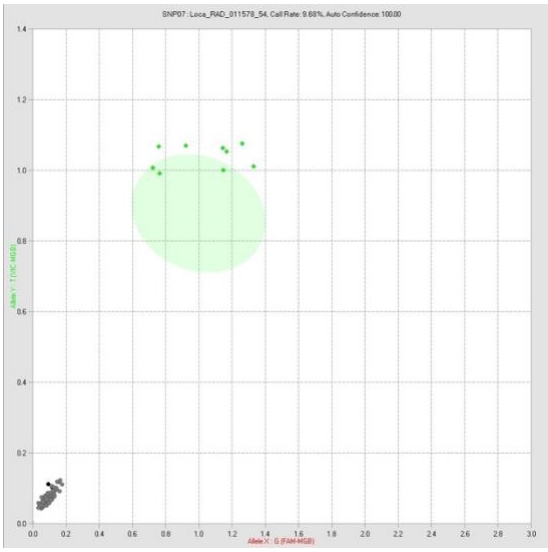
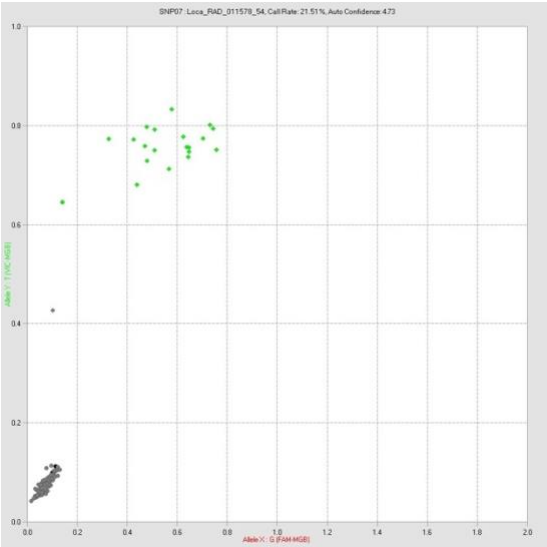
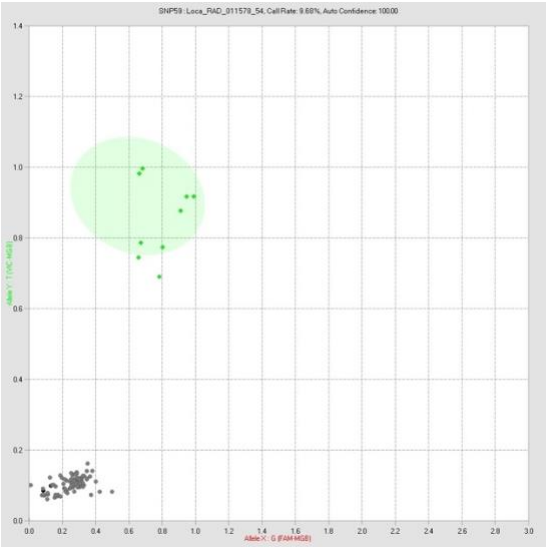
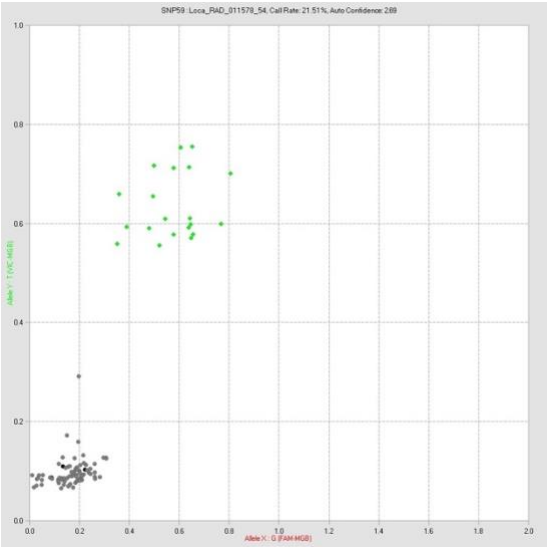
appears fixed. No/little separation between samples that fail and samples that worked.
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP05	Loca_RAD_018476_68

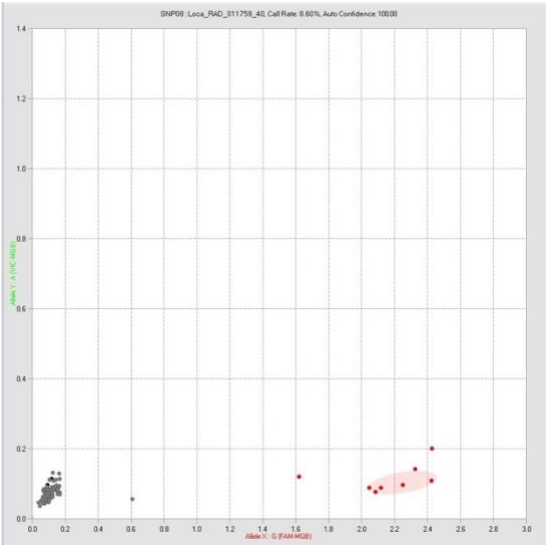
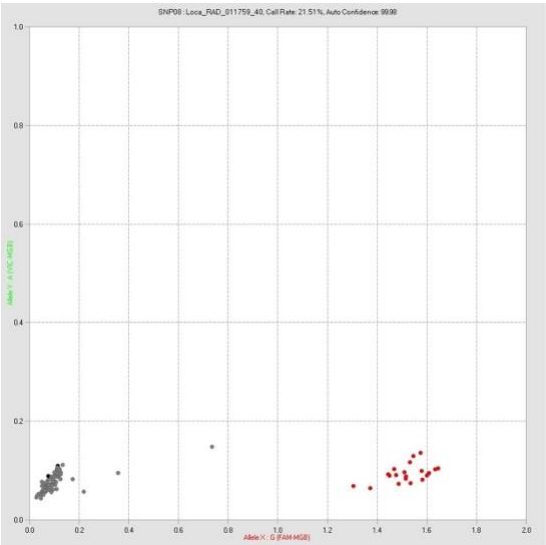
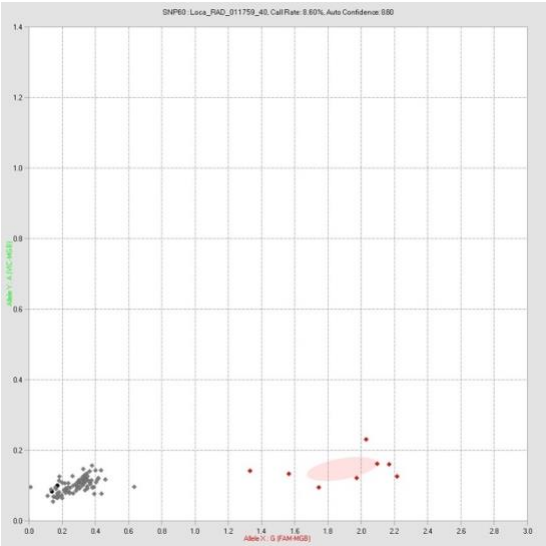
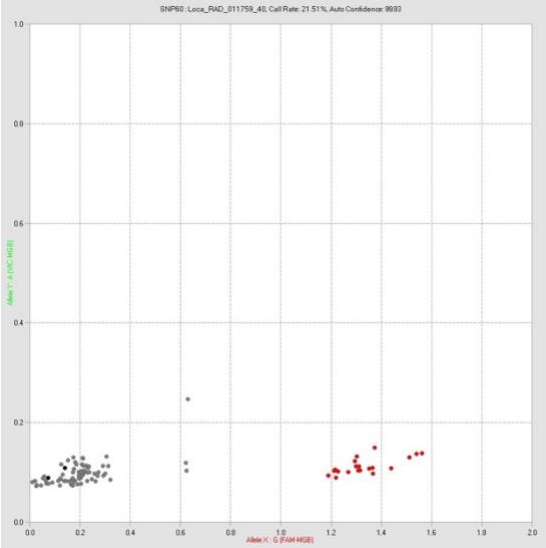
appears nearly fixed, high skew
category yellow (use with caution)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP06	Loca_RAD_011081_47

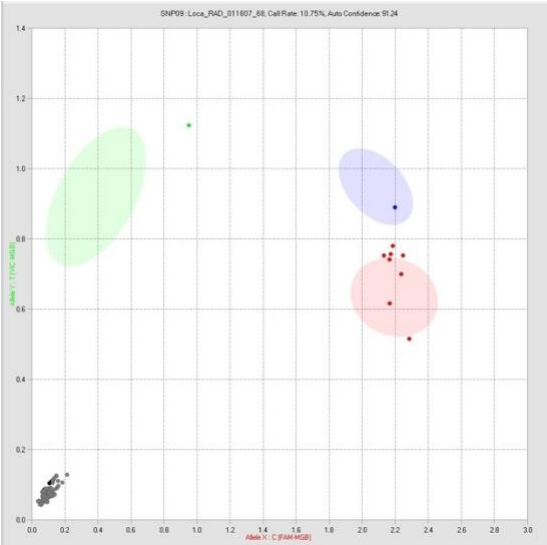
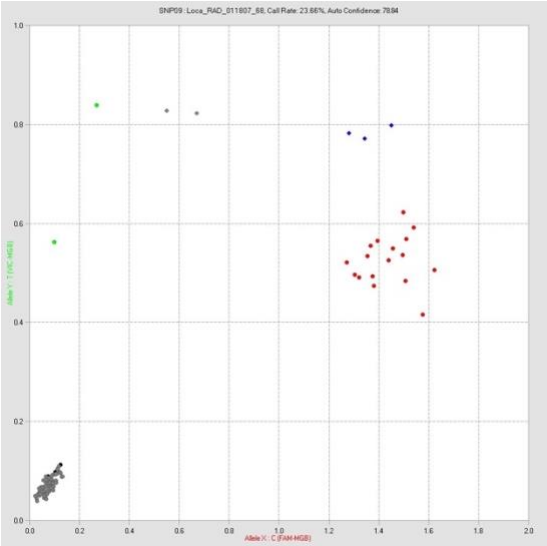
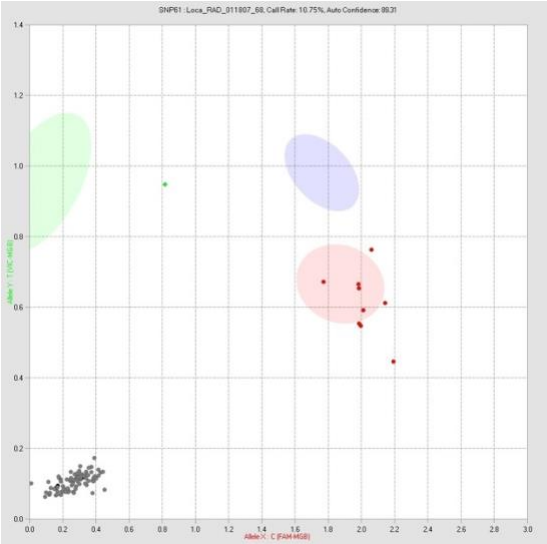
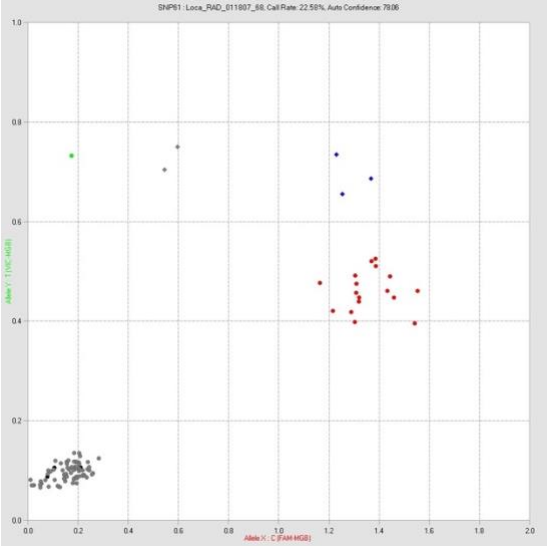
appears nearly fixed, high skew. Weird distribution of genotypes (more heterozygotes than I'd expect) category yellow (use with caution)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP07	Loca_RAD_011578_54

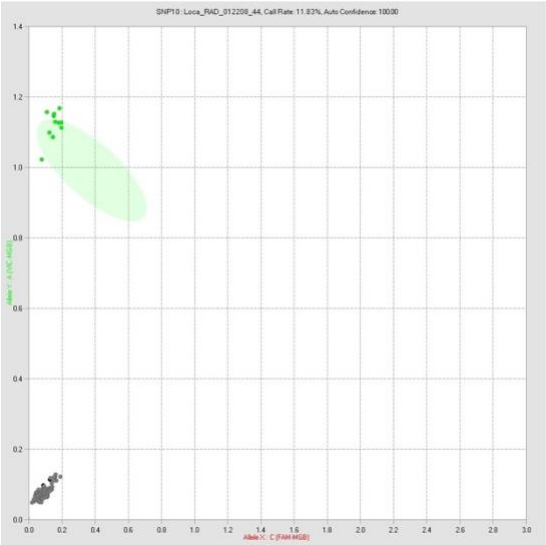
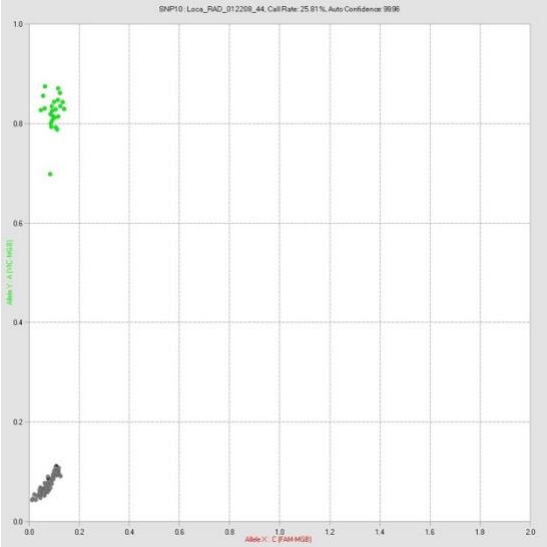
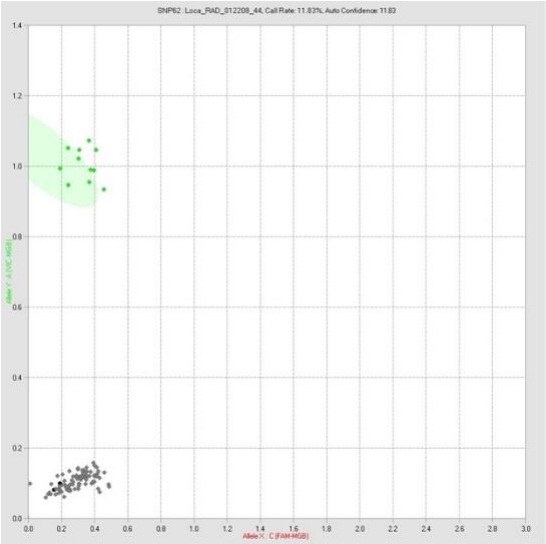
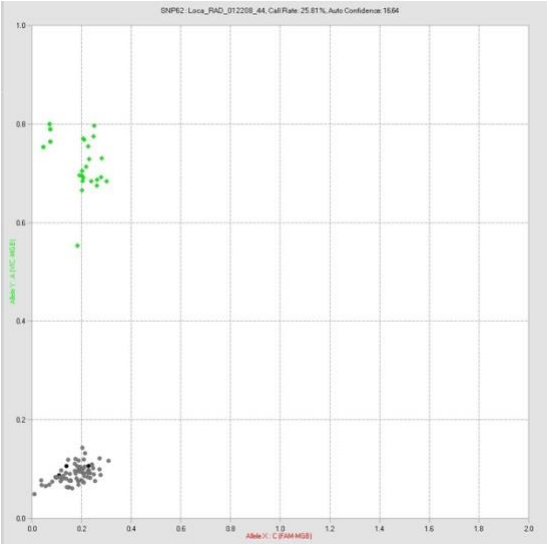
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP08	Loca_RAD_011759_40

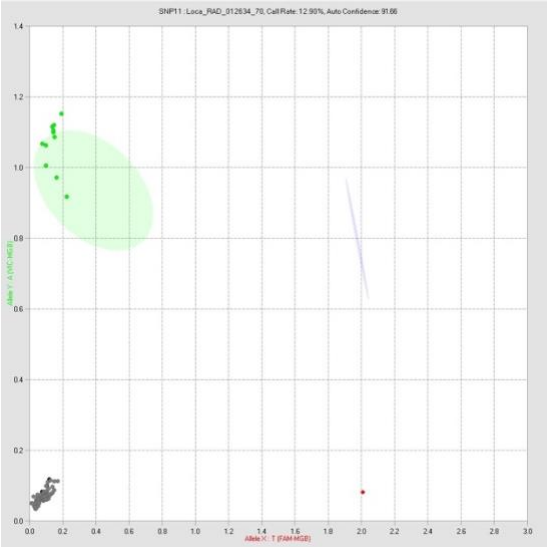
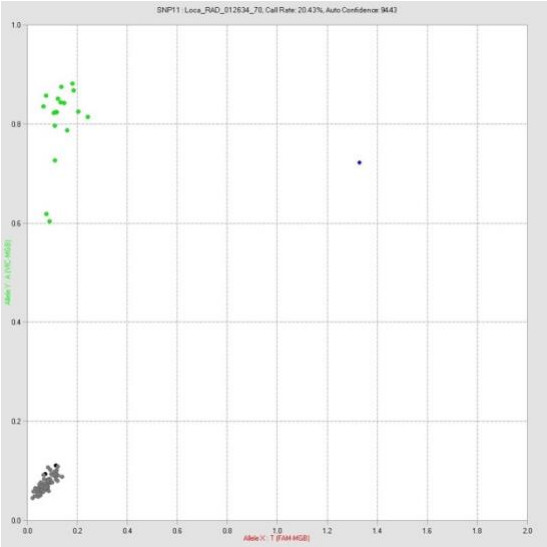
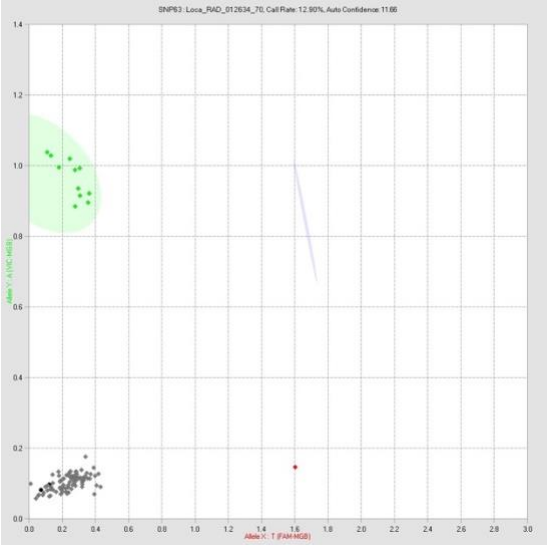
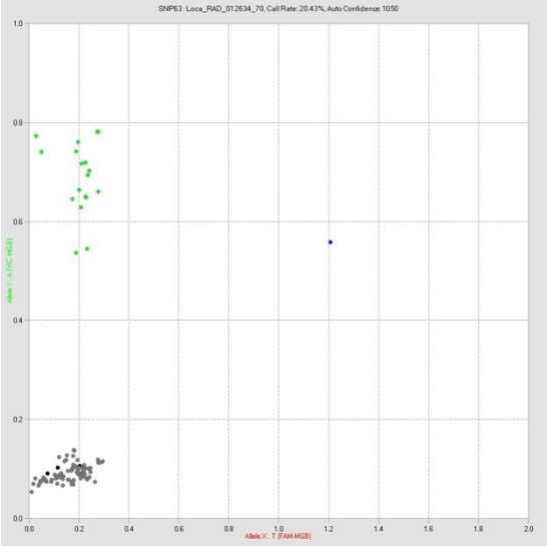
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP09	Loca_RAD_011807_68

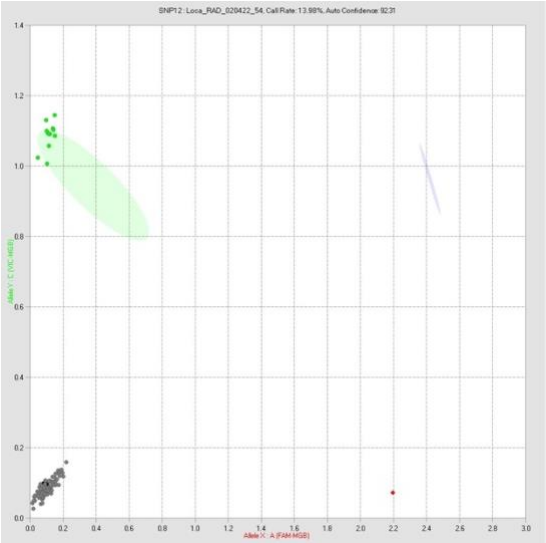
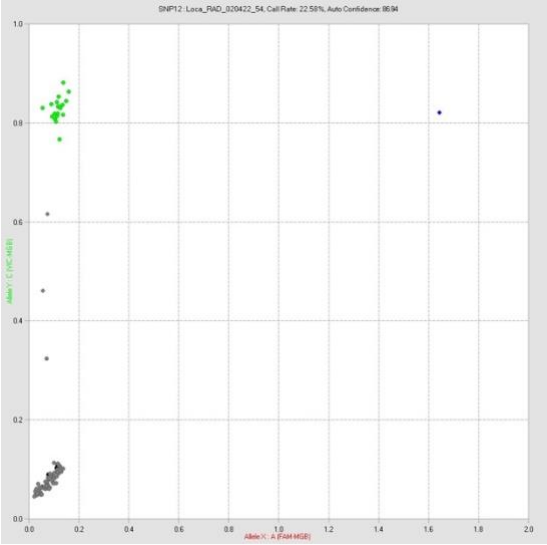
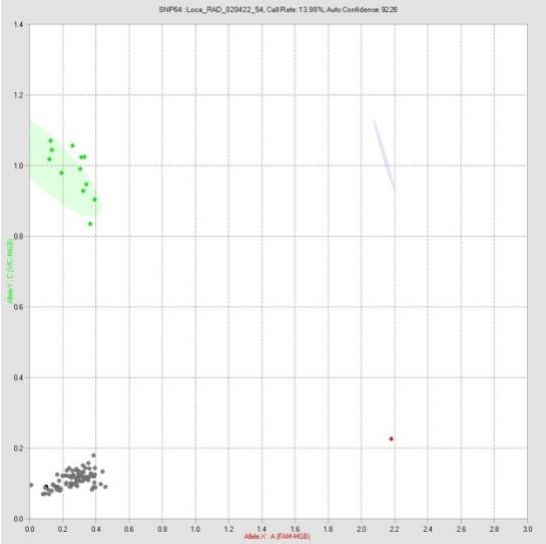
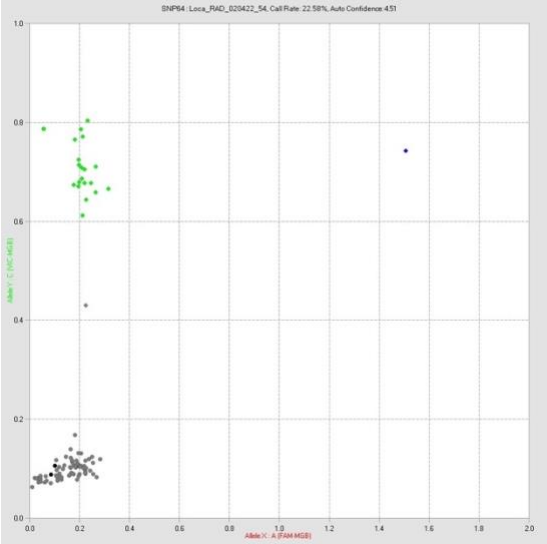
some samples fail, but 3 distinct groupings. Heterozygotes and FAM homozygotes very close, may be hard to score on chips with few heterozygotes (like Chip 1—not 100% confident in that one heterozygote in the upper screenshot). 1 replicate mismatch (sample 23)
category yellow (use with caution)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP10	Loca_RAD_012208_44

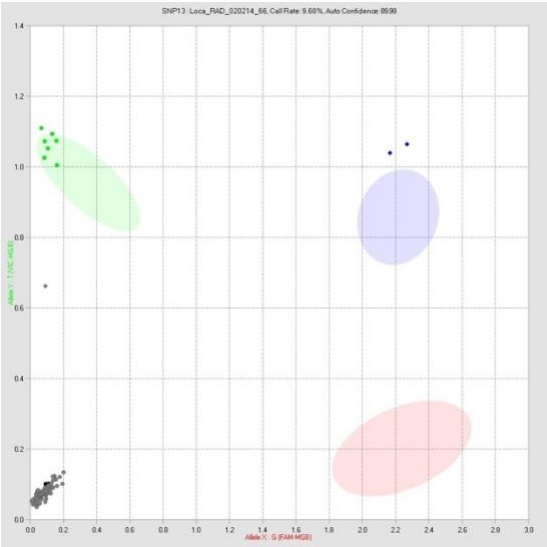
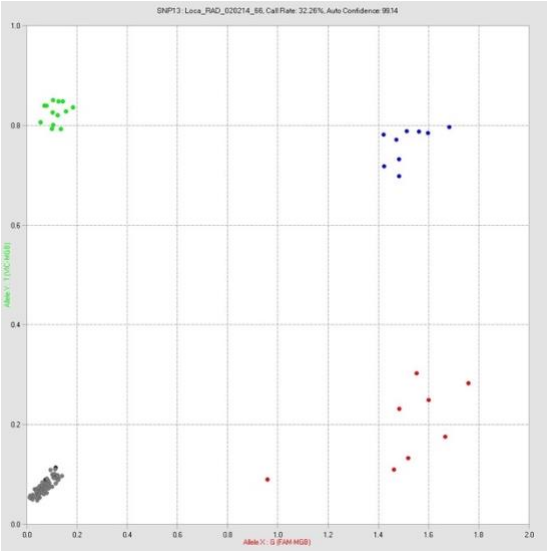
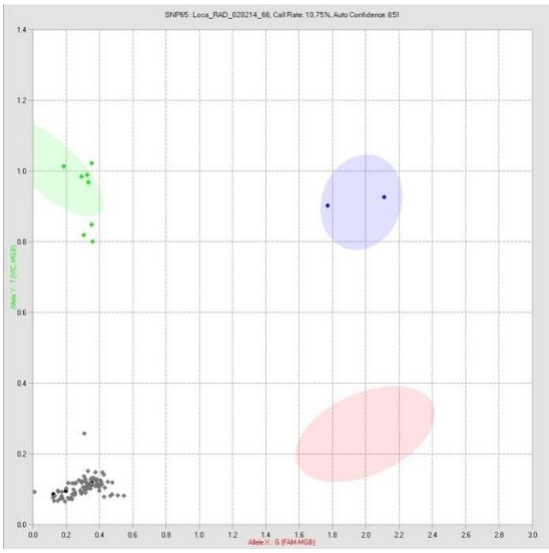
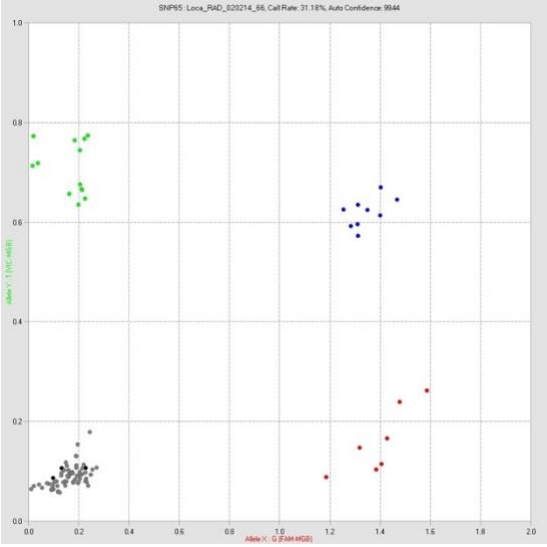
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP11	Loca_RAD_012634_70

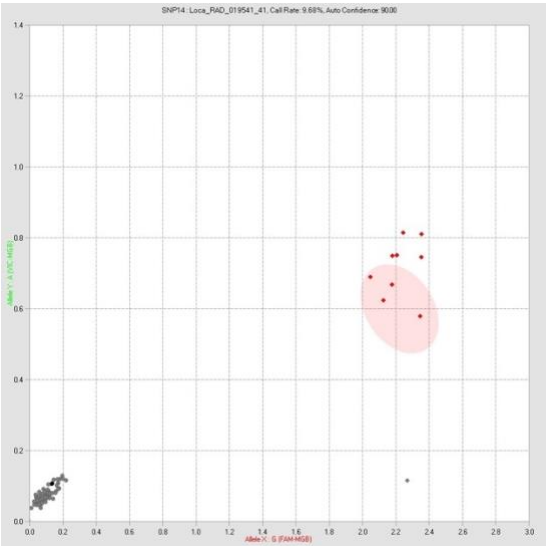
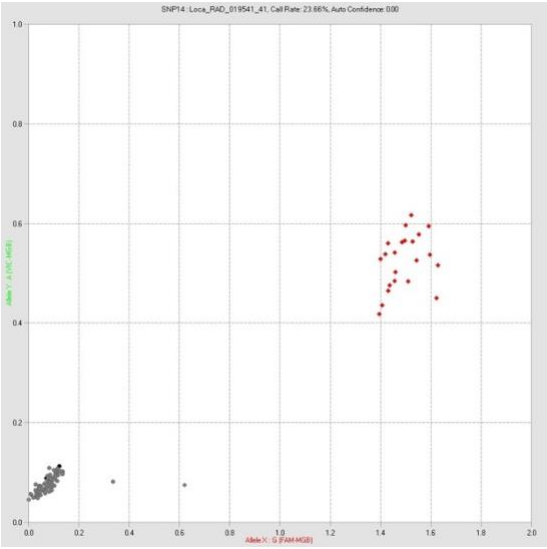
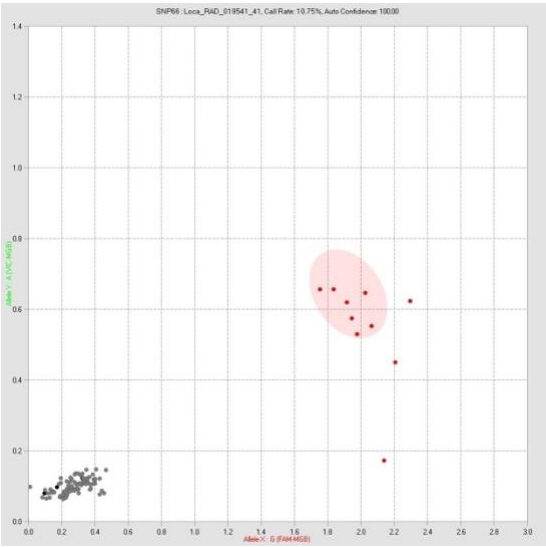
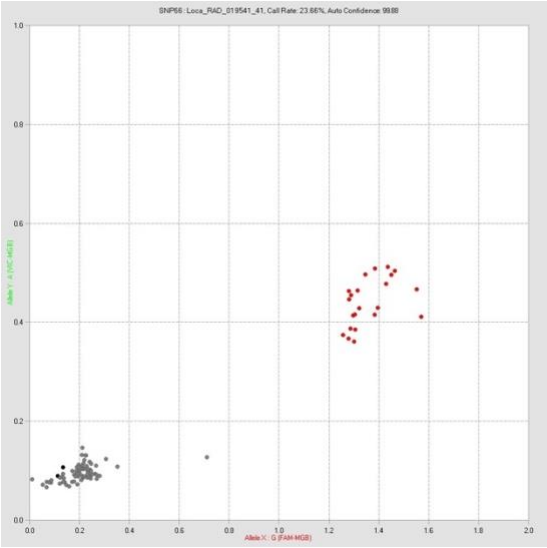
appears fixed, some outliers
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP12	Loca_RAD_020422_54

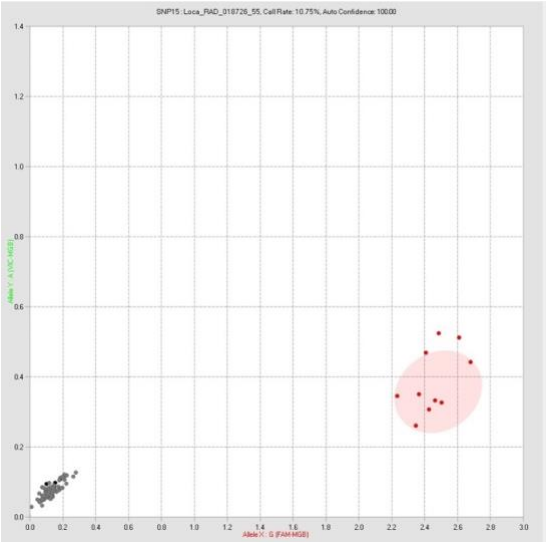
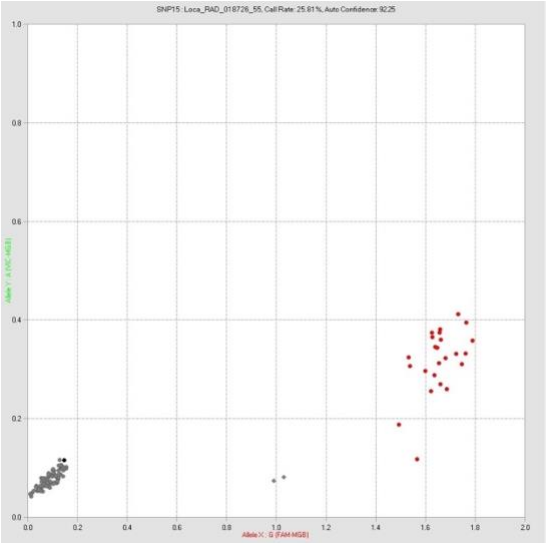
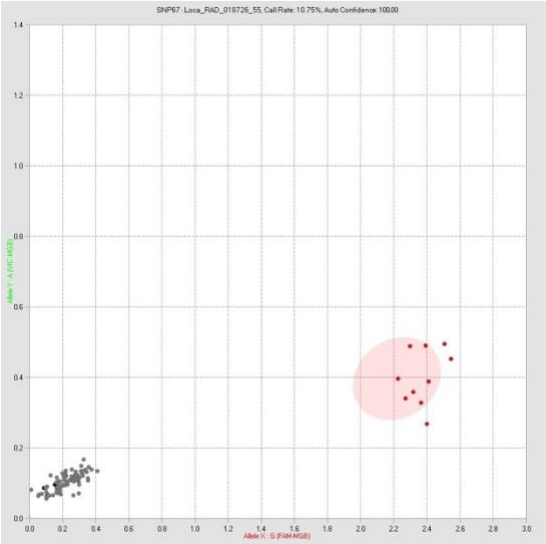
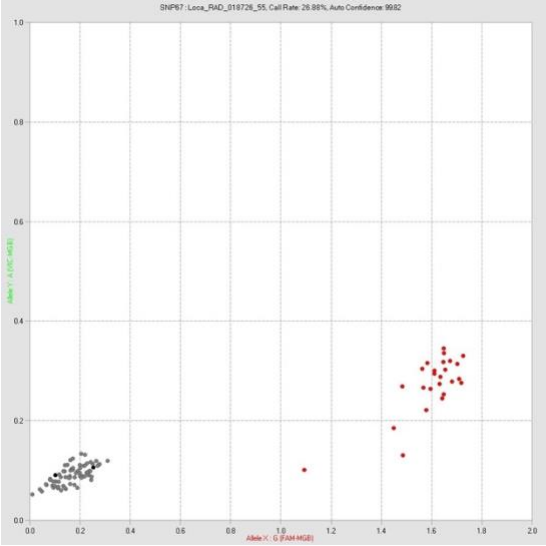
appears fixed, some outliers
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP13	Loca_RAD_020214_66

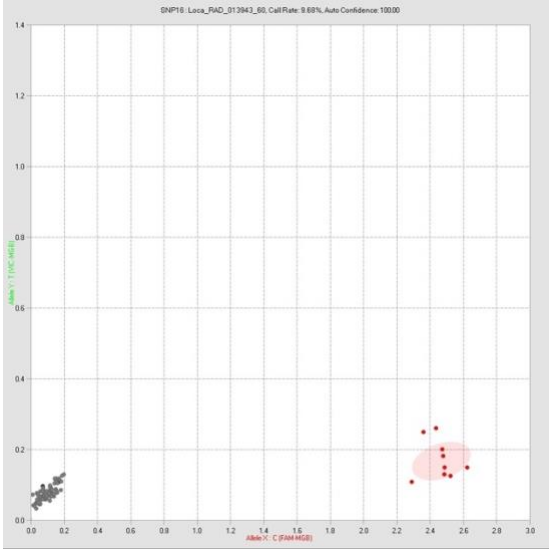
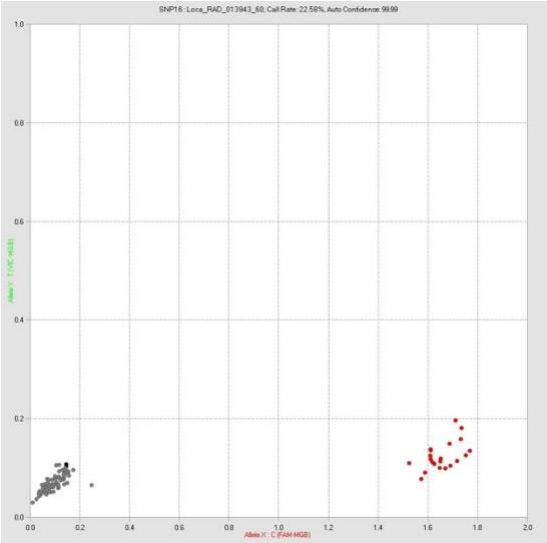
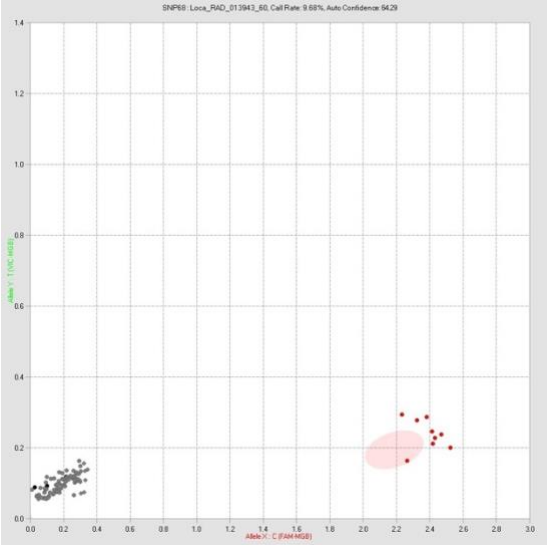
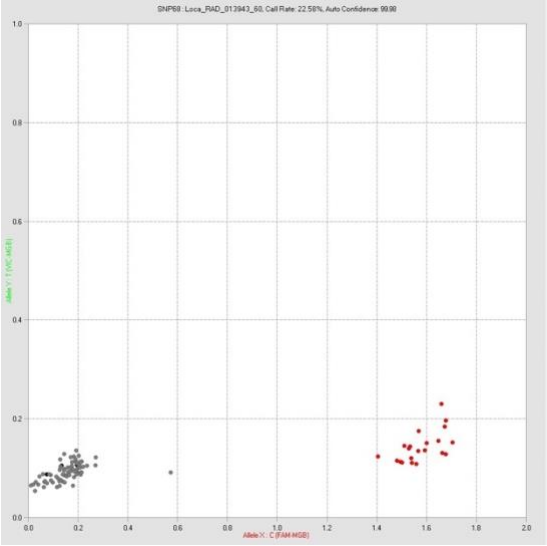
3 clearly distinct groupings
category green (ok to use)
1 replicate mismatch (sample 40)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP14	Loca_RAD_019541_41

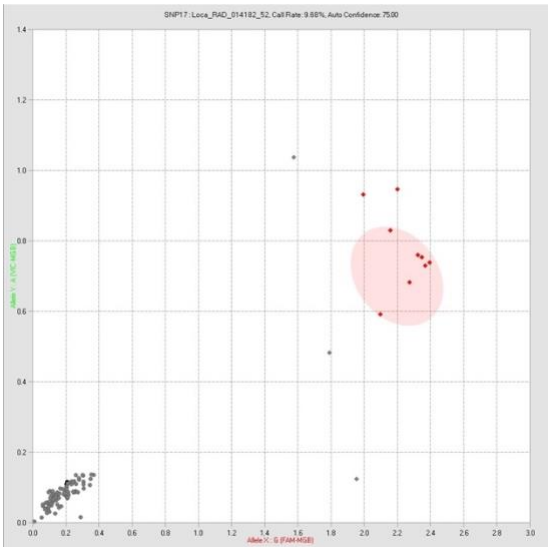
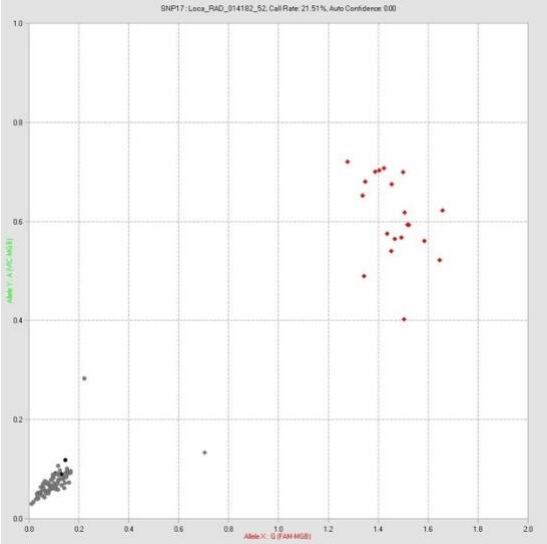
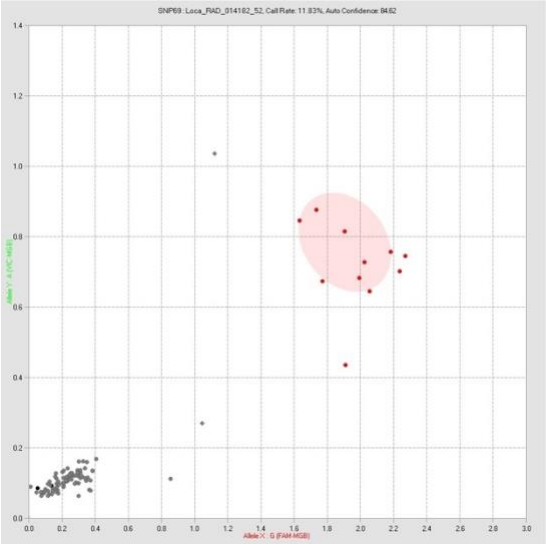
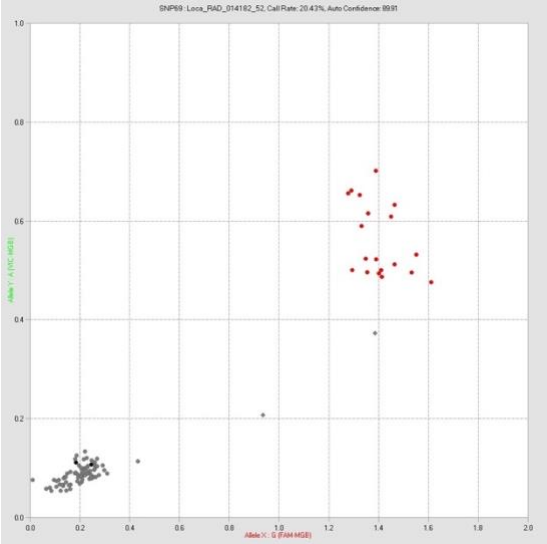
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP15	Loca_RAD_018726_55

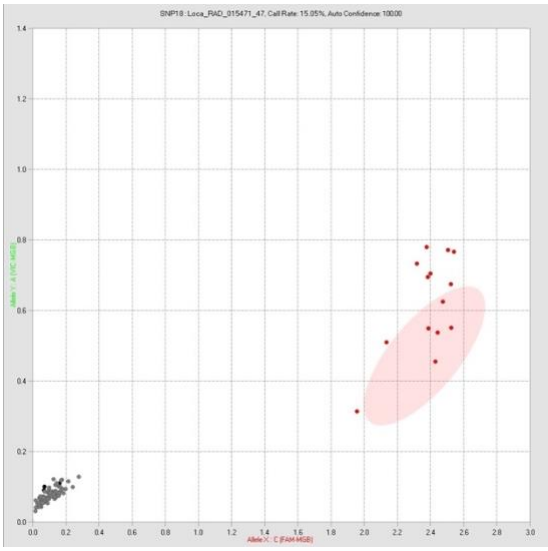
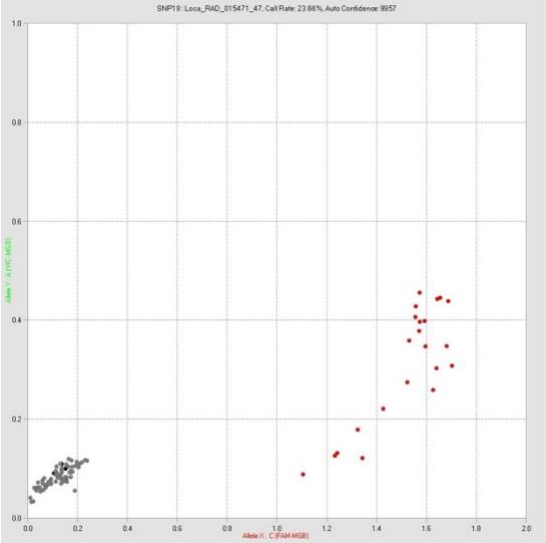
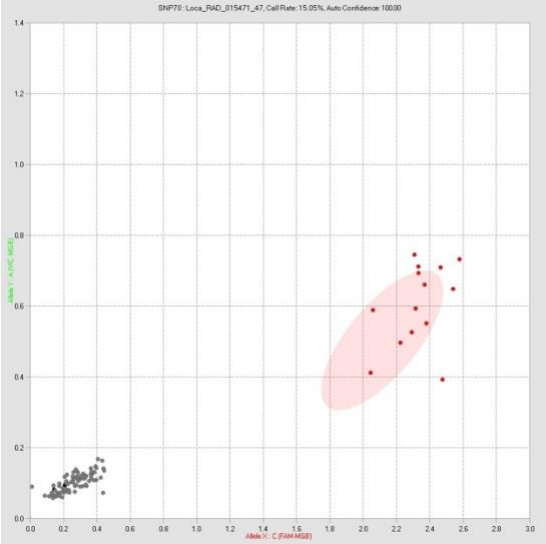
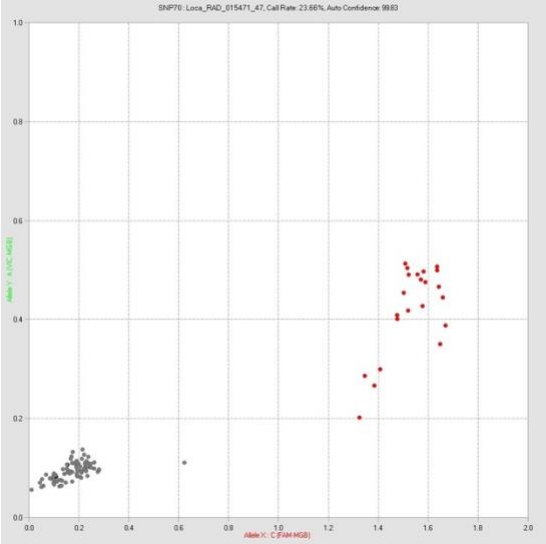
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP16	Loca_RAD_013943_60

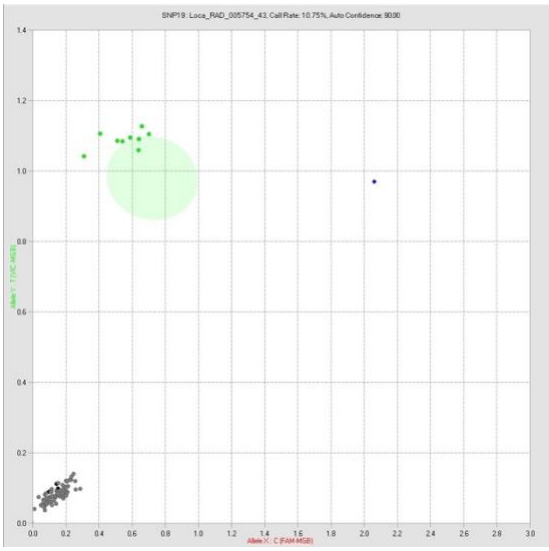
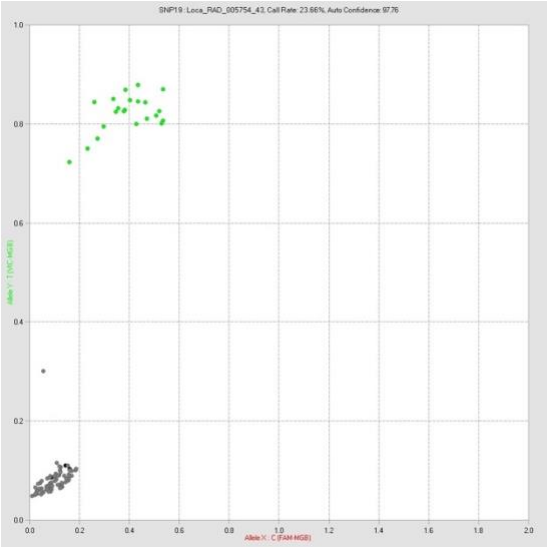
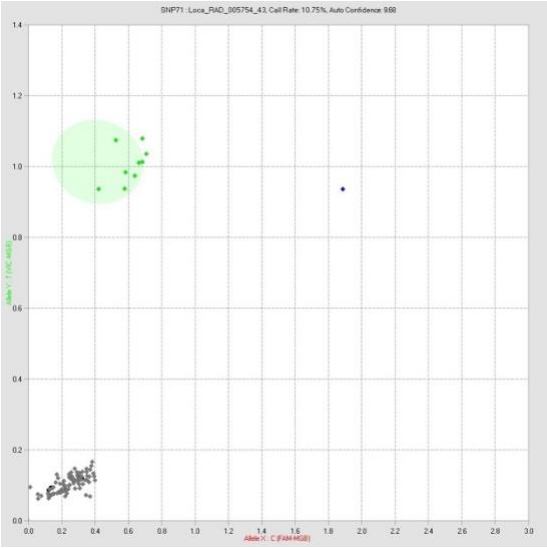
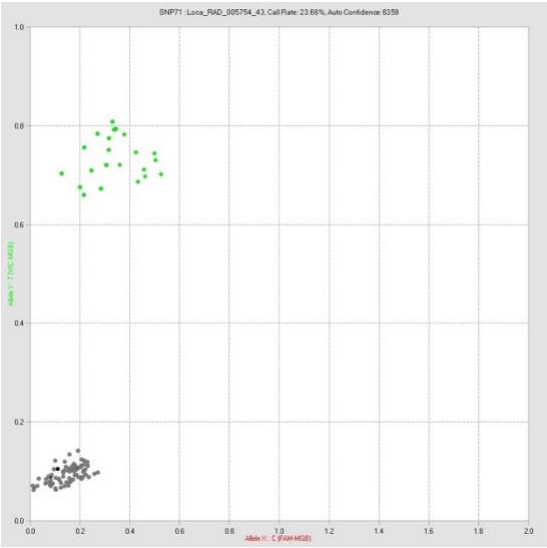
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP17	Loca_RAD_014182_52

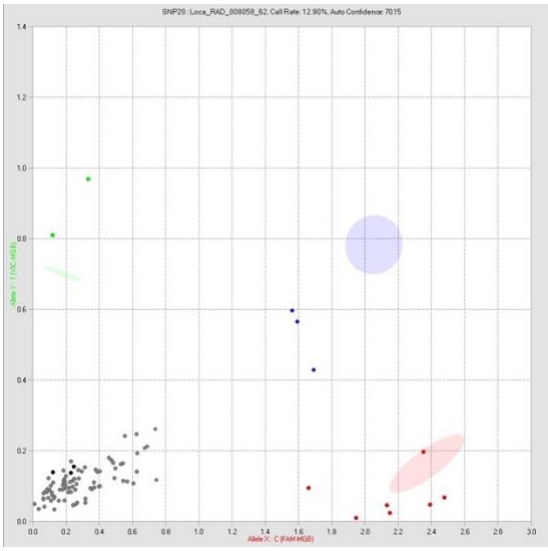
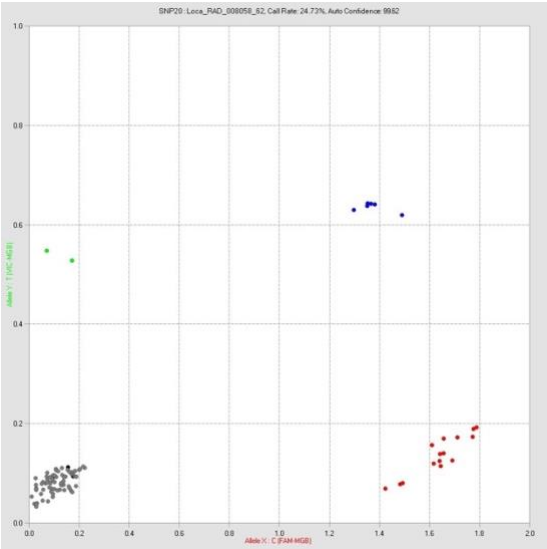
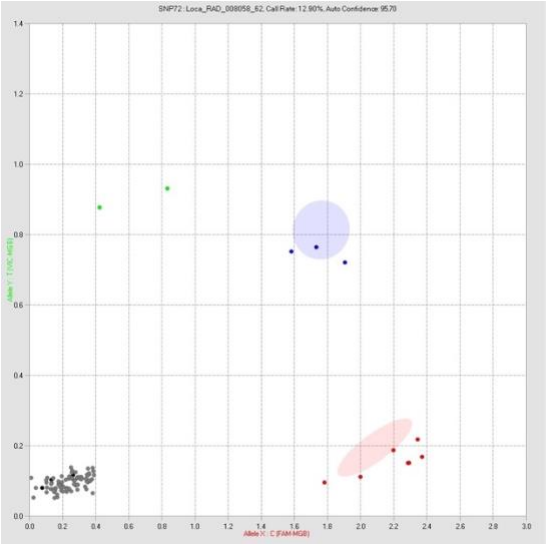
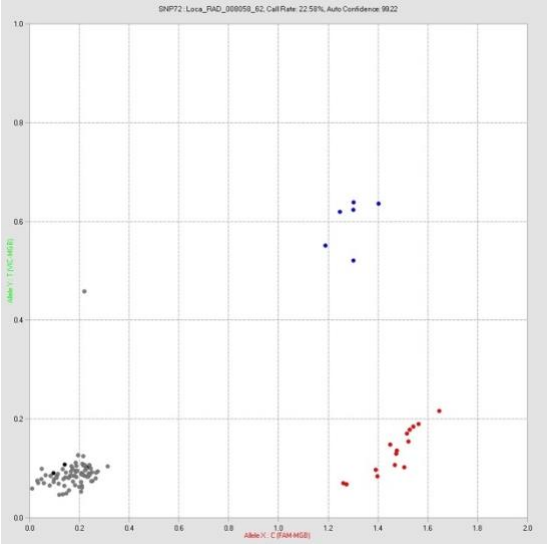
appears fixed, some outliers. Weird cluster shape and position—it's possible these are a heterozygote and homozygote cluster that have merged, but hard to tell and would have no idea where the cutoff between the two clusters would be, so this is probably the best way to score these.
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP18	Loca_RAD_015471_47

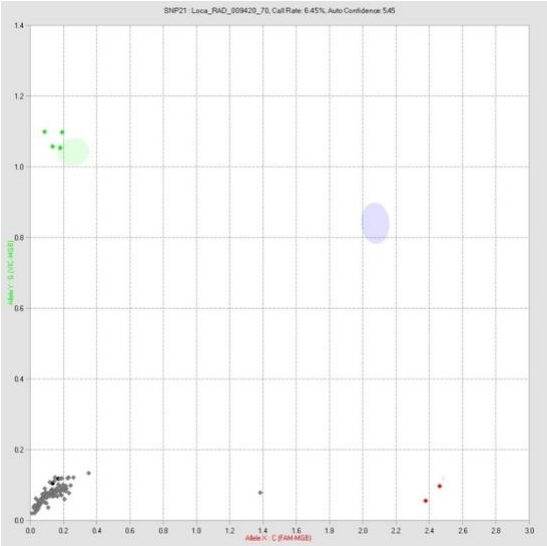
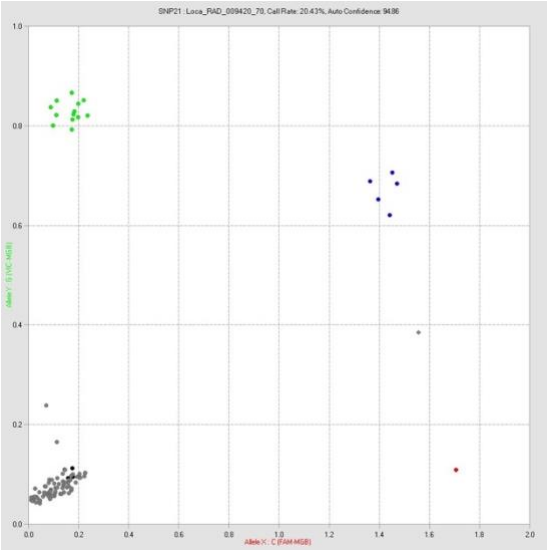
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP19	Loca_RAD_005754_43

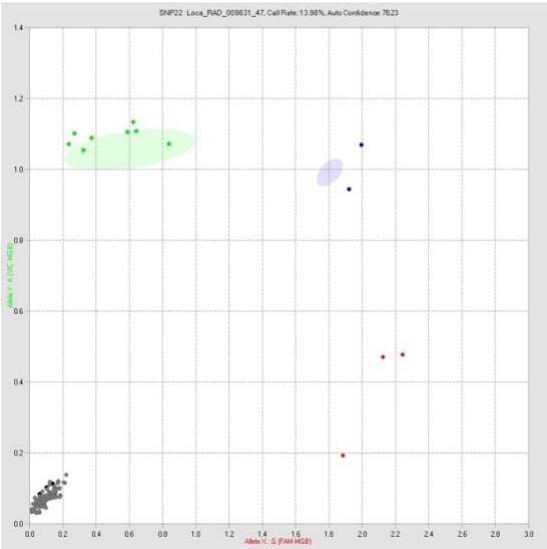
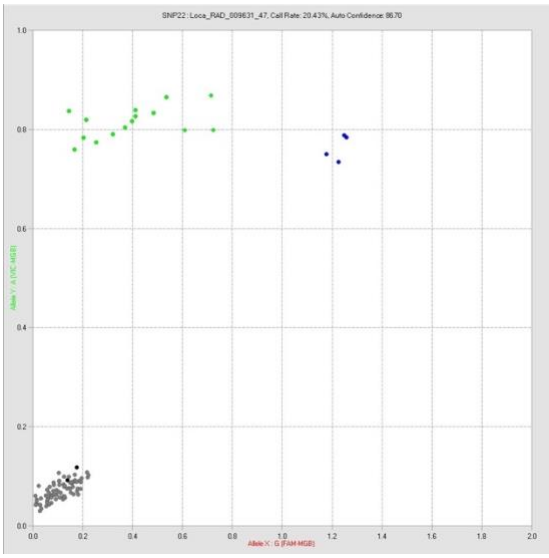
appears fixed, some outliers
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP20	Loca_RAD_008058_62

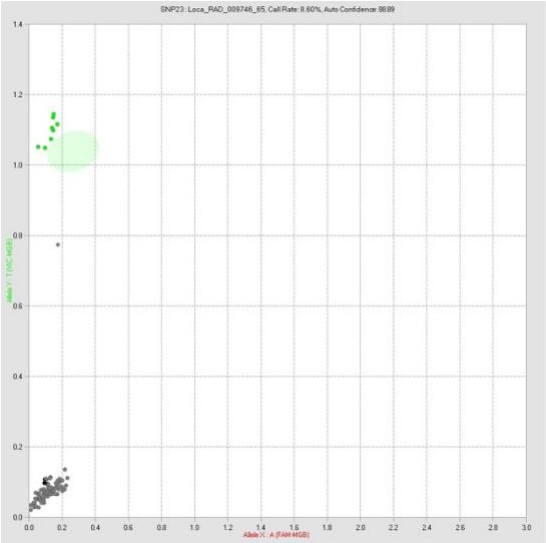
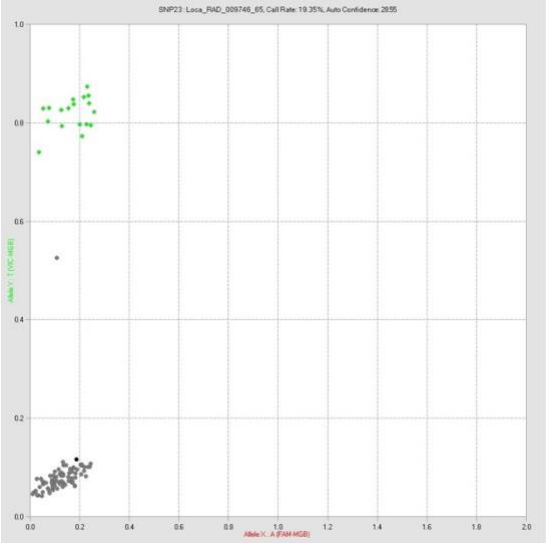
3 clearly distinct groupings
category green (ok to use)
1 replicate mismatch (sample 198)

Chip 1	Chip 2
	
SNP21	Loca_RAD_009420_70

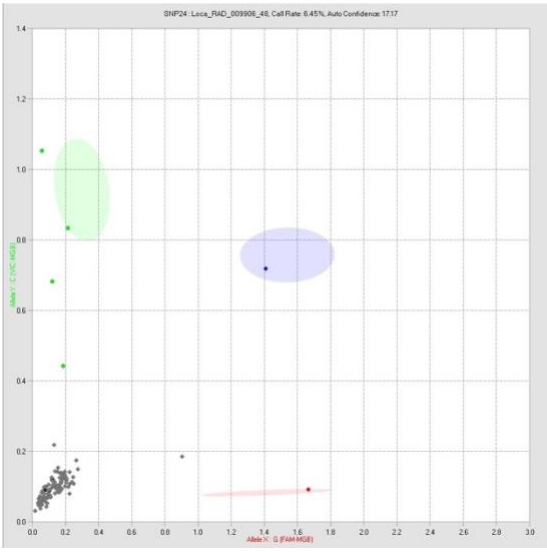
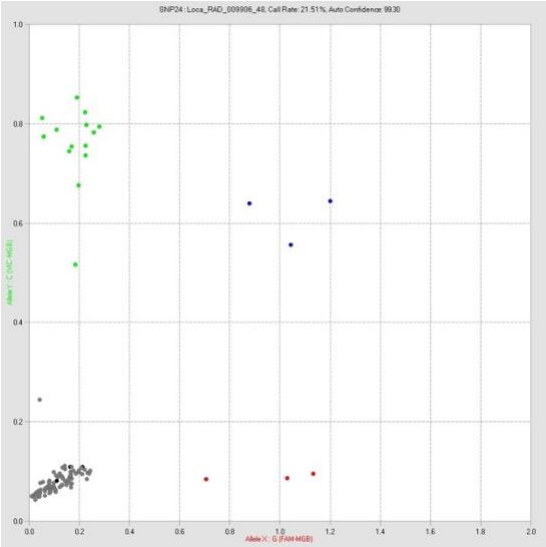
many samples fail, but 3 distinct groupings
category yellow (use with caution)

Chip 1	Chip 2
	
SNP22	Loca_RAD_009631_47

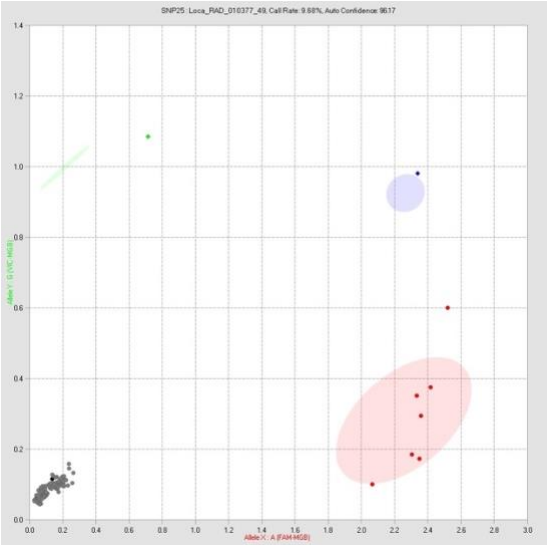
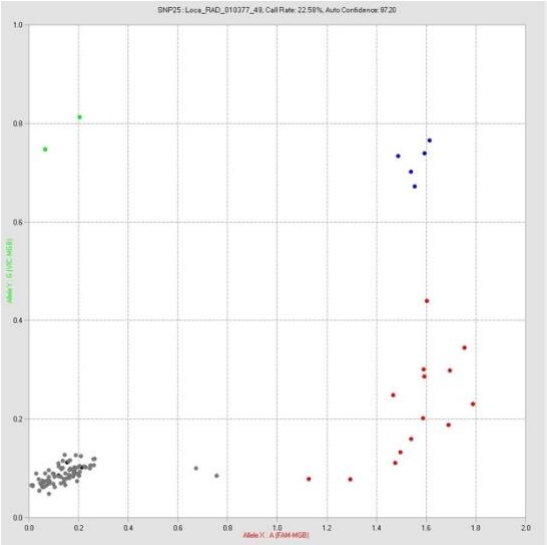
some samples fail, but 3 distinct groupings. HEX (VIC on axes) homozygotes smear towards the heterozygote cluster—may be due to sample quality or could just be a quirk of the assay. Shouldn't affect scoring as there seems to be a good amount of space between the homozygotes and heterozygotes, but may be confusing if you don't observe another cluster with this one on a chip run.
category yellow (use with caution)

Chip 1	Chip 2
	
SNP23	Loca_RAD_009746_65

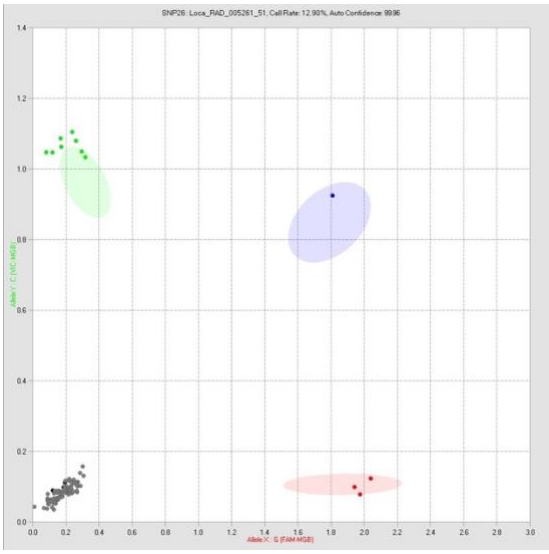
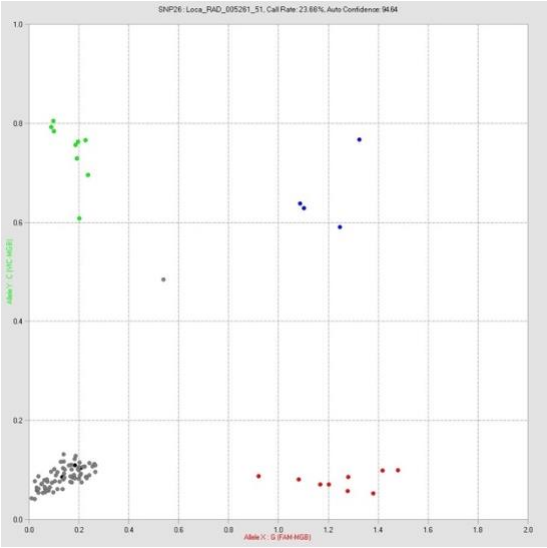
appears fixed
category gray (do not use)

Chip 1	Chip 2
	
SNP24	Loca_RAD_009906_48

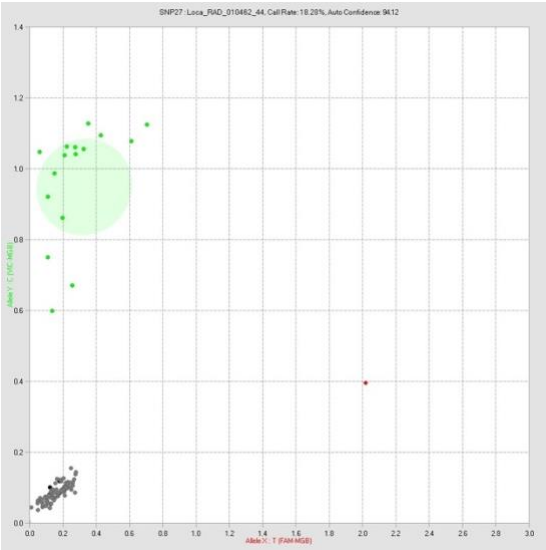
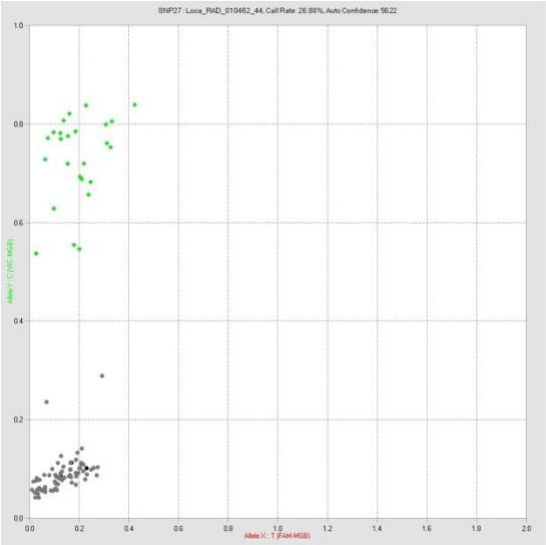
some samples fail, but 3 distinct groupings
category yellow (use with caution)

Chip 1	Chip 2
	
SNP25	Loca_RAD_010377_49

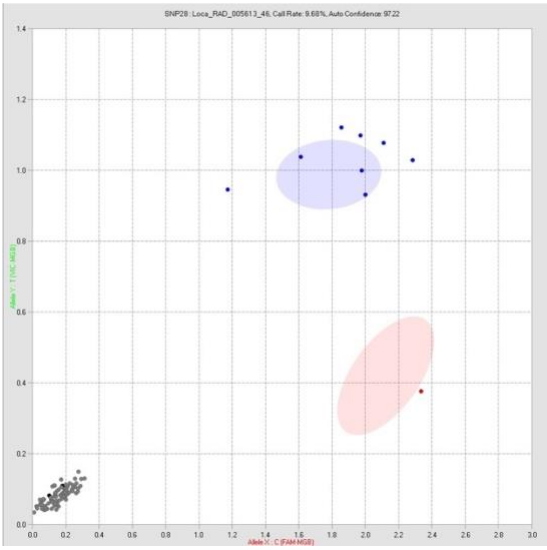
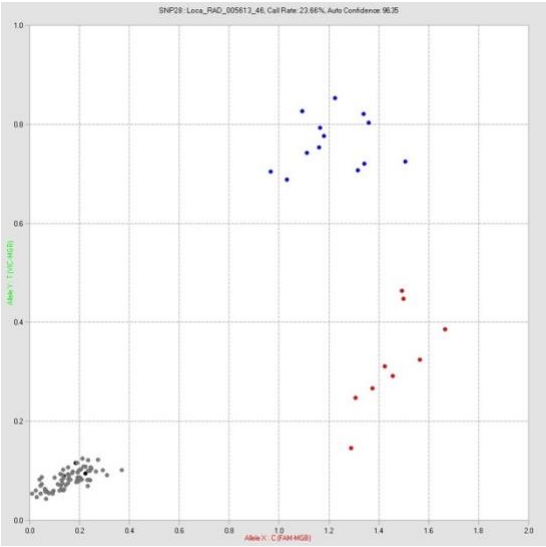
some samples fail, but 3 distinct groupings. FAM homozygotes smear towards the heterozygote cluster—may be due to sample quality or could just be a quirk of the assay. Shouldn't affect scoring as there seems to be a good amount of space between the homozygotes and heterozygotes, but may be confusing if you don't observe another cluster with this one on a chip run.
category green (ok to use)

Chip 1	Chip 2
	
SNP26	Loca_RAD_005261_51

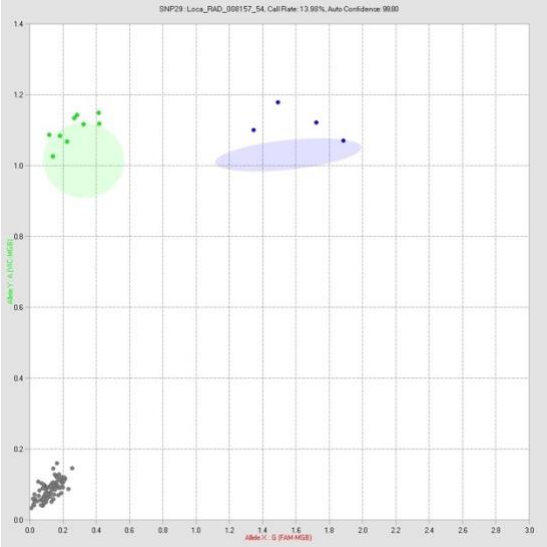
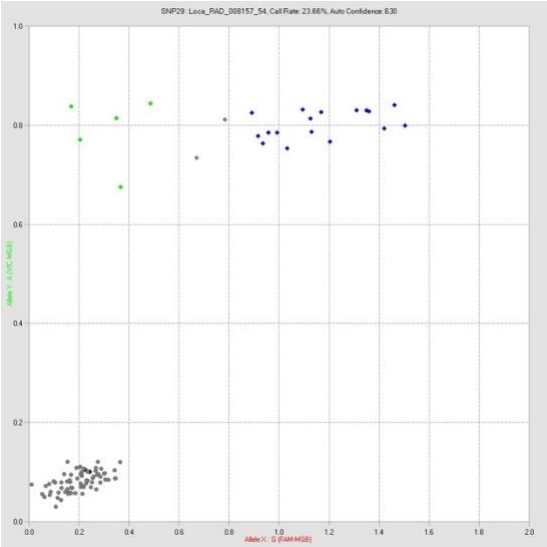
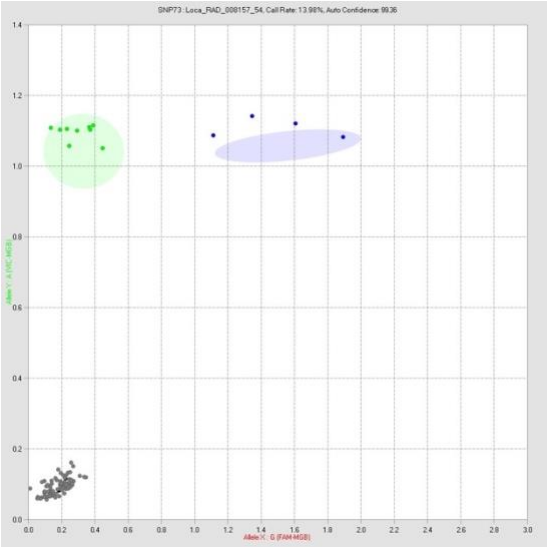
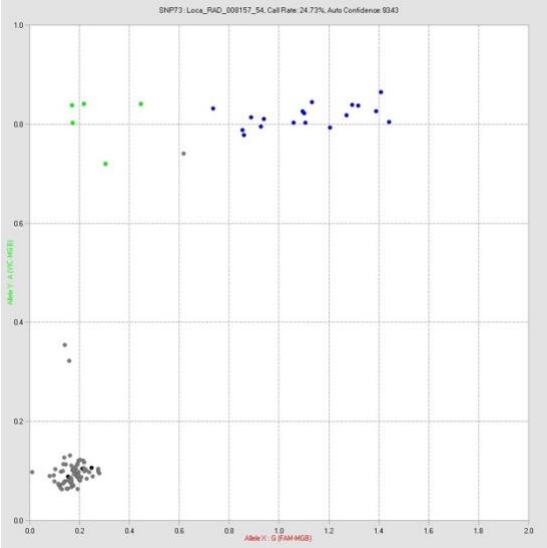
some samples fail, but 3 distinct groupings –
category green (ok to use)

Chip 1	Chip 2
	
SNP27	Loca_RAD_010462_44

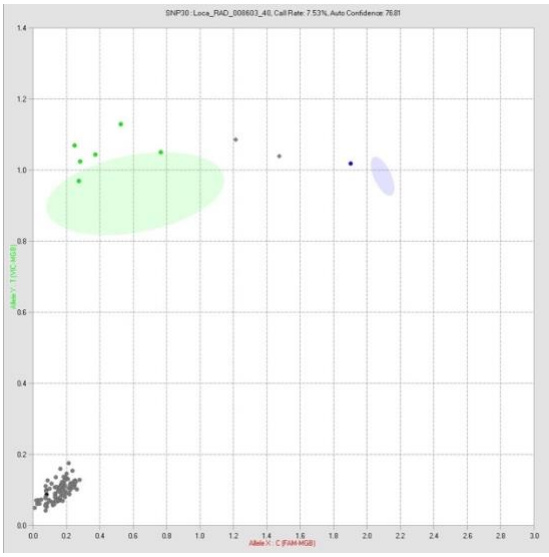
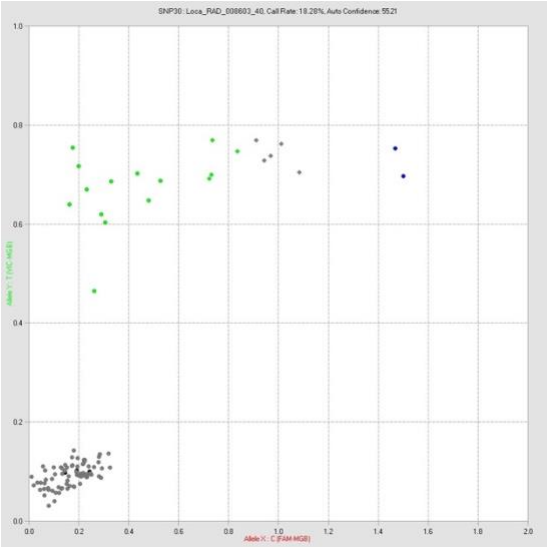
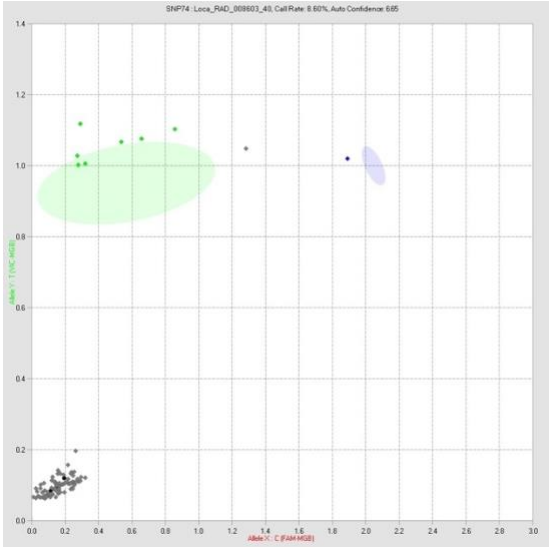
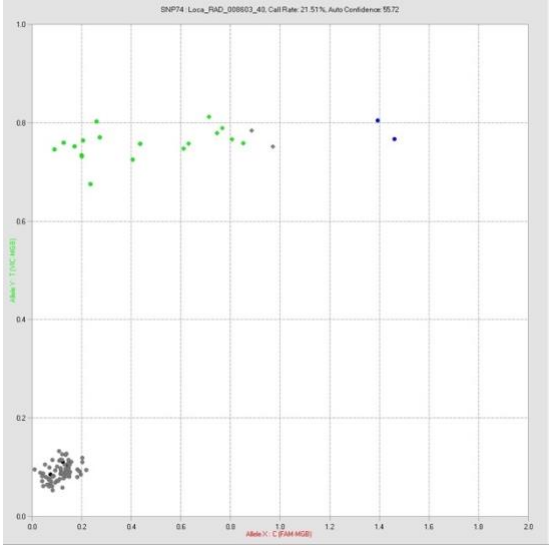
appears fixed
category gray (do not use)

Chip 1	Chip 2
	
SNP28	Loca_RAD_005613_46

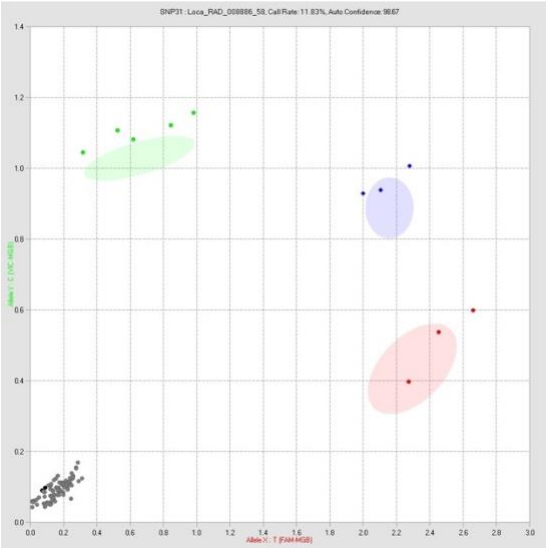
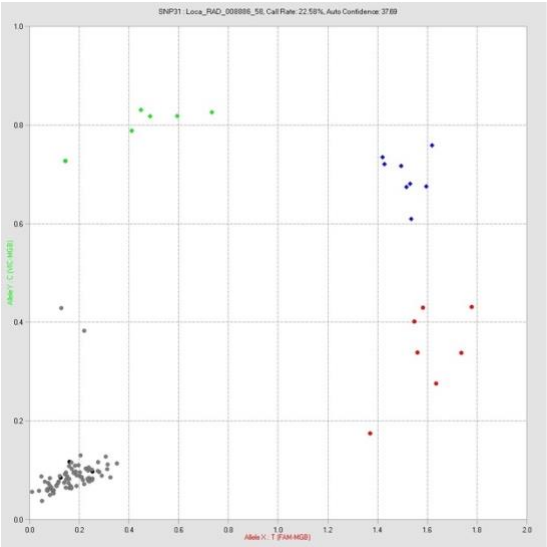
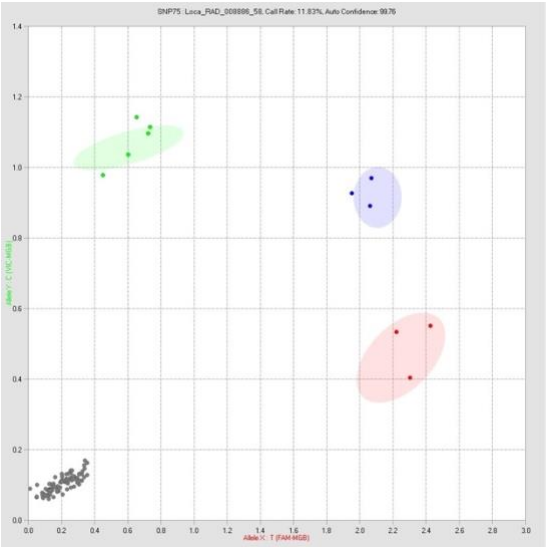
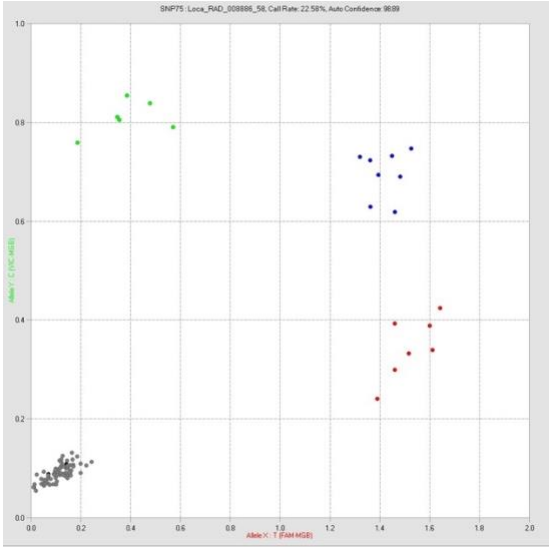
strange distribution, only two distinct groupings? More heterozygotes than I'd expect and sort of loose clusters that might indicate that the heterozygote cluster may be a merge heterozygote and homozygote cluster.
category yellow (use with caution)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP29	Loca_RAD_008157_54

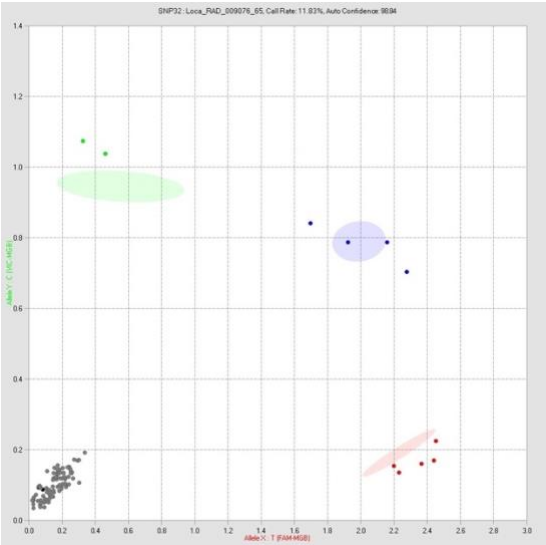
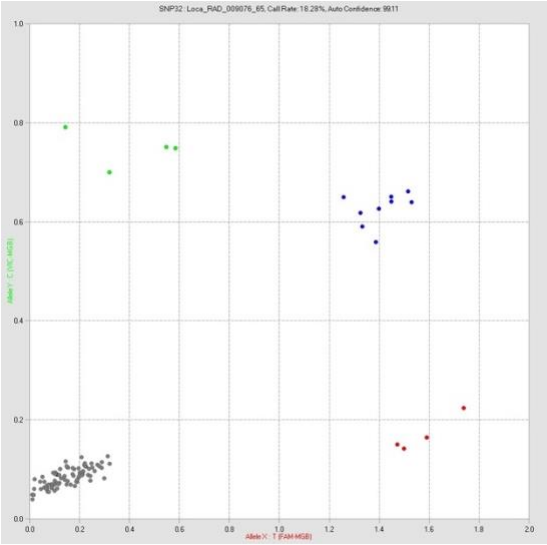
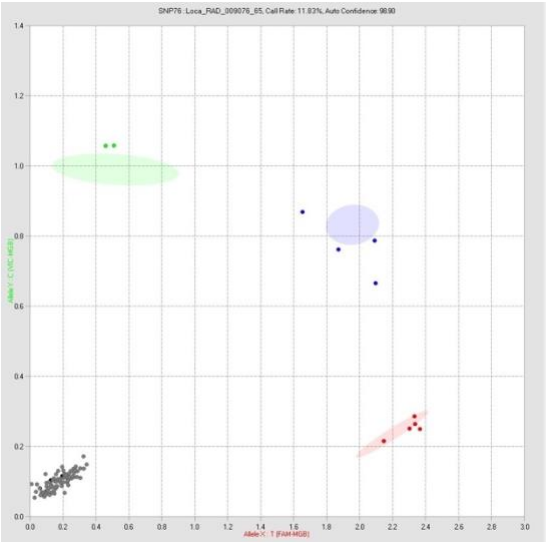
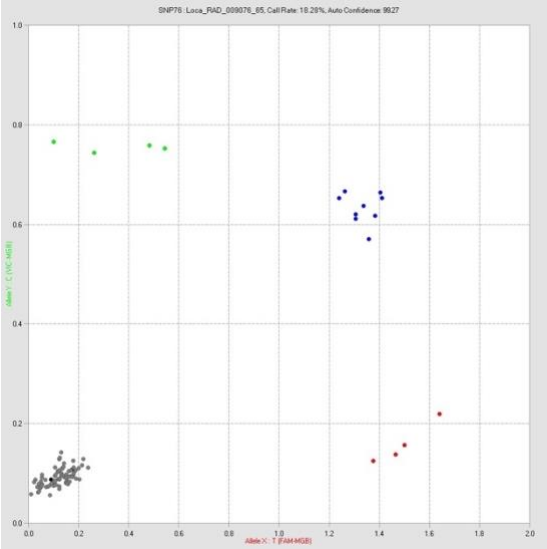
strange distribution, only two groupings? Heterozygotes and HEX (VIC on axes) smear towards each other making it difficult to tell where one ends and the other begins—genotype calls may be incorrect (particularly on chip 2) category yellow (use with caution)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP30	Loca_RAD_008603_40

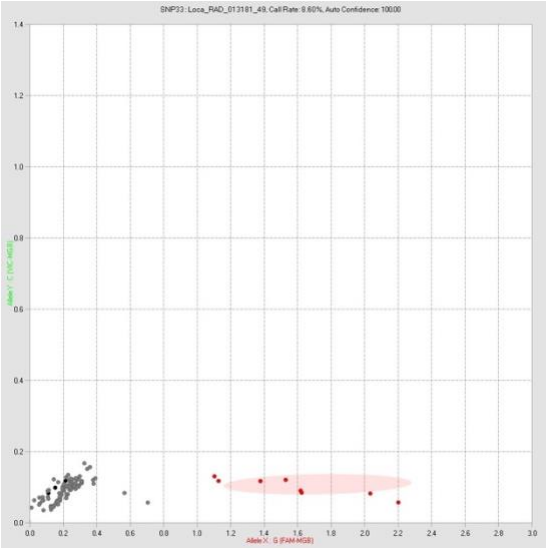
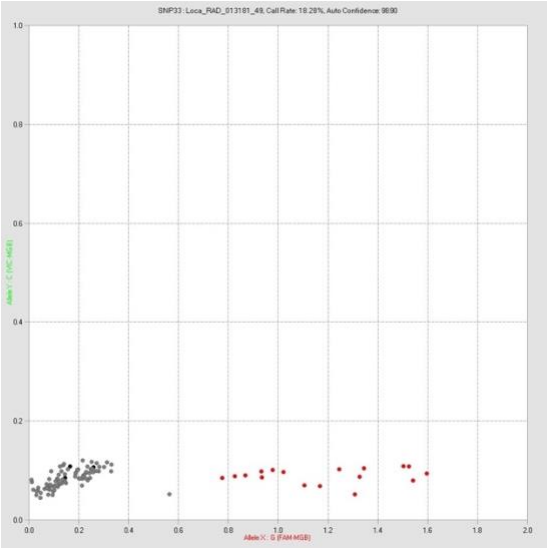
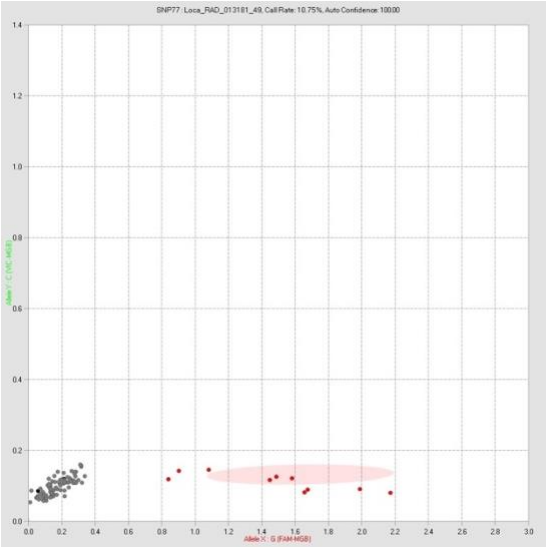
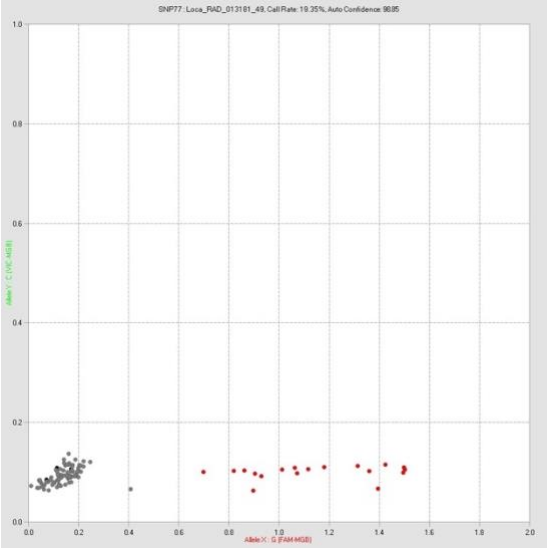
strange distribution, only two groupings? Heterozygotes and HEX (VIC on axes) smear towards each other making it difficult to tell where one ends and the other begins.
category yellow (use with caution)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP31	Loca_RAD_008886_58

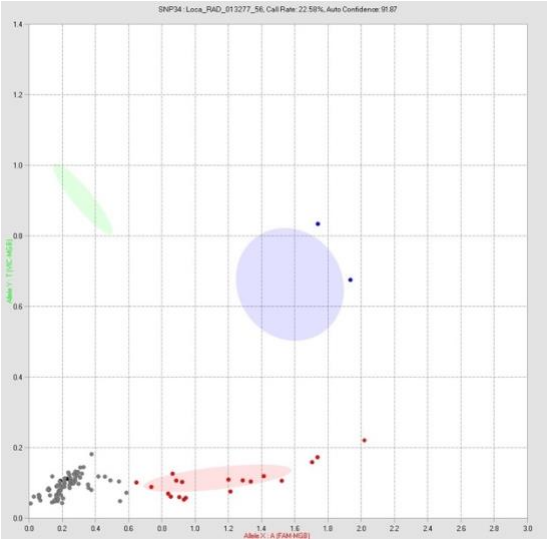
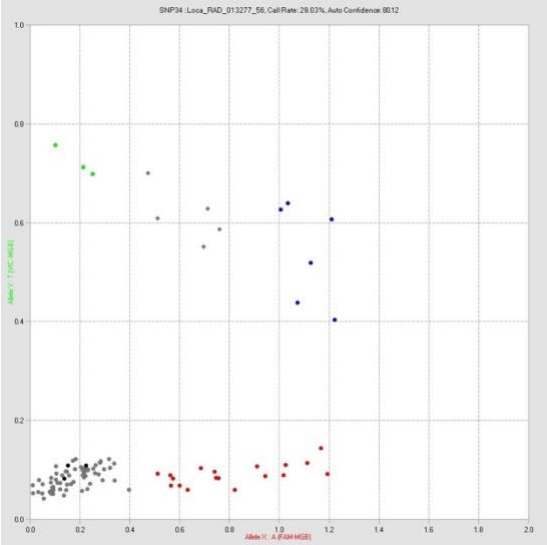
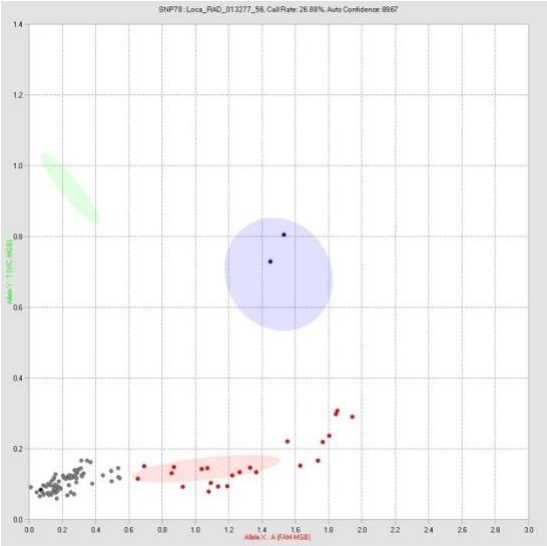
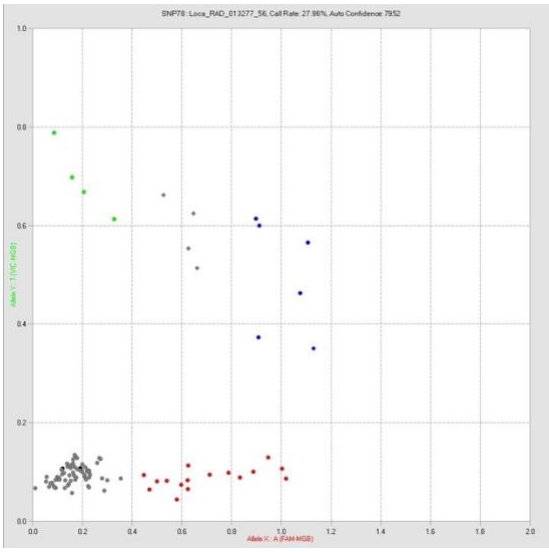
3 clearly distinct groupings
category green (ok to use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP32	Loca_RAD_009076_65

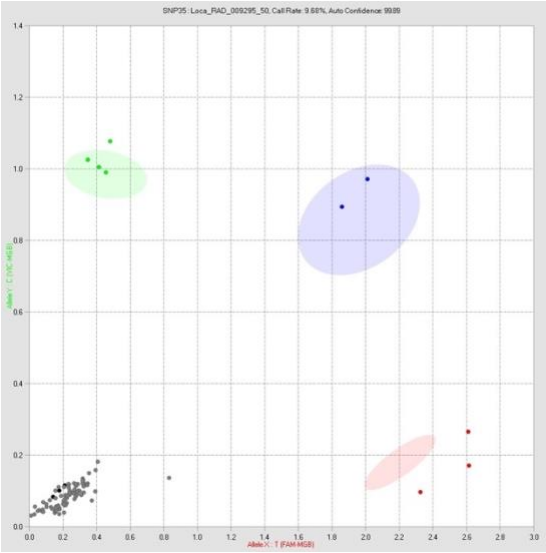
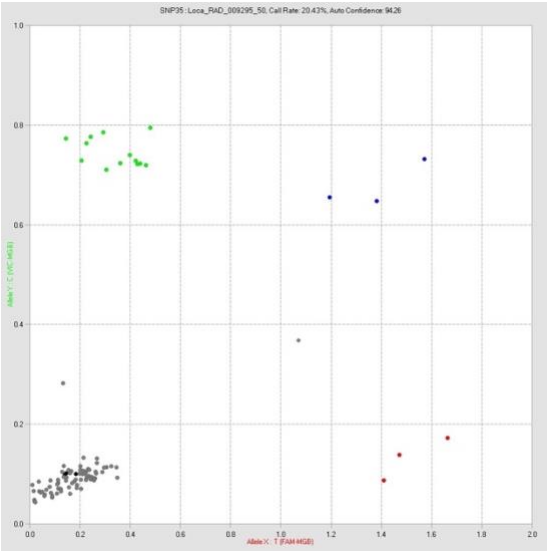
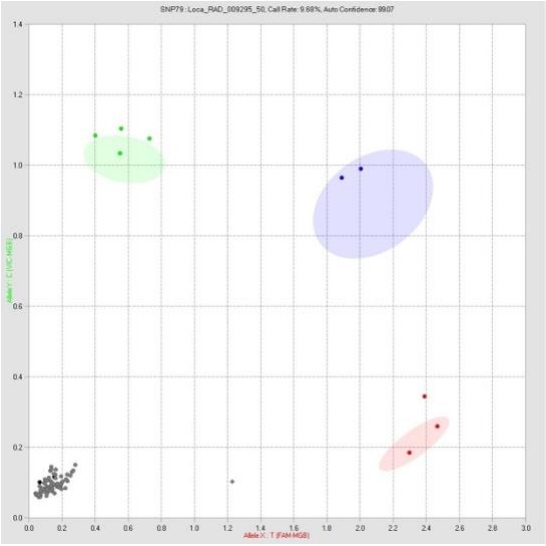
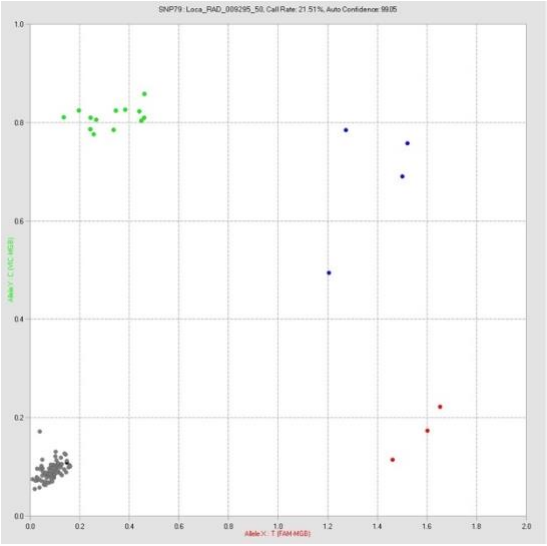
3 clearly distinct groupings
category green (ok to use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP33	Loca_RAD_013181_49

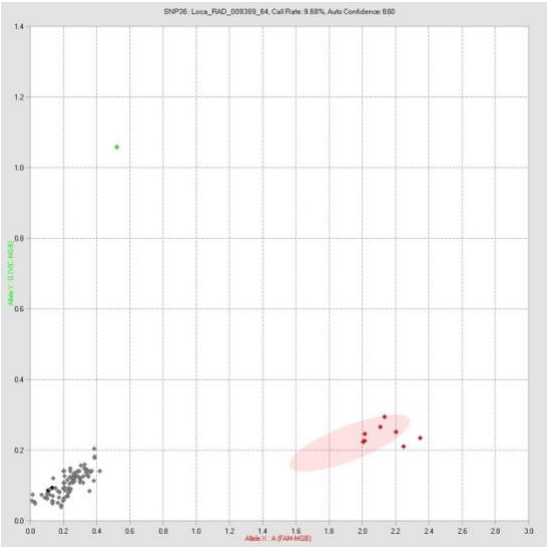
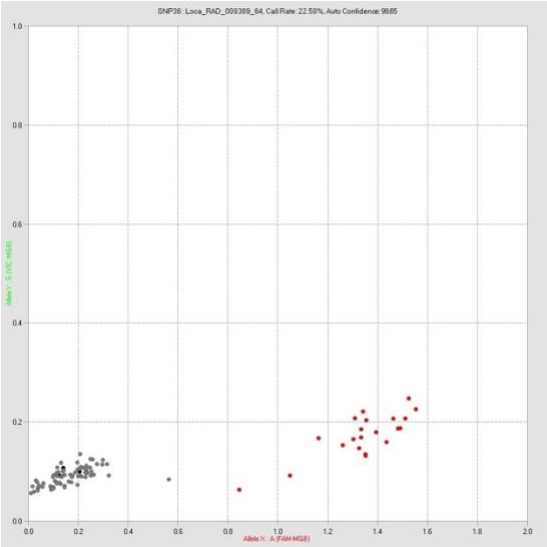
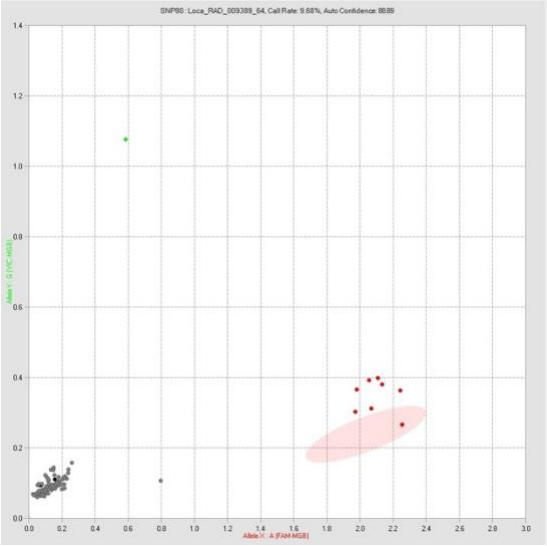
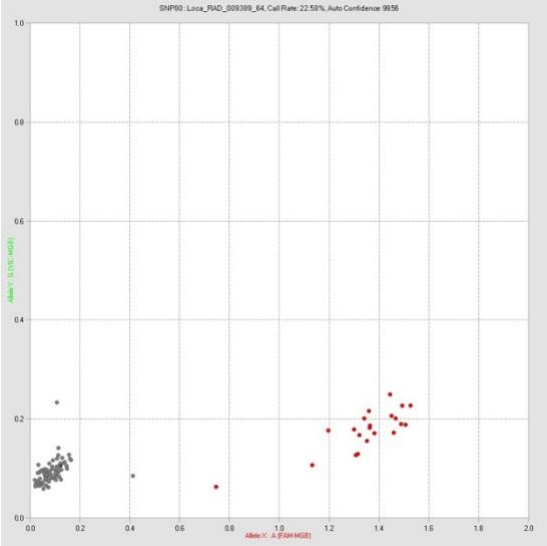
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP34	Loca_RAD_013277_56

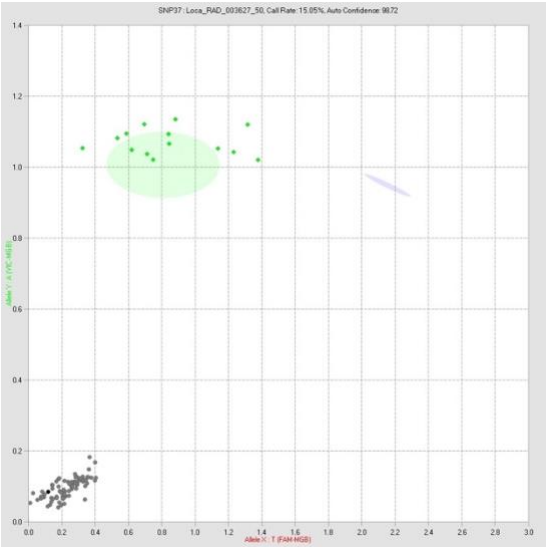
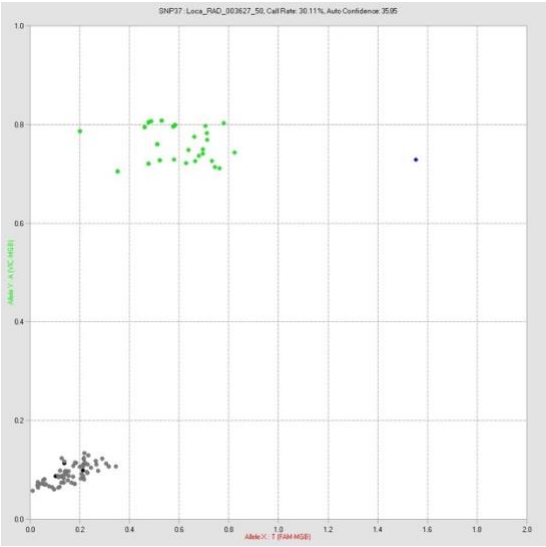
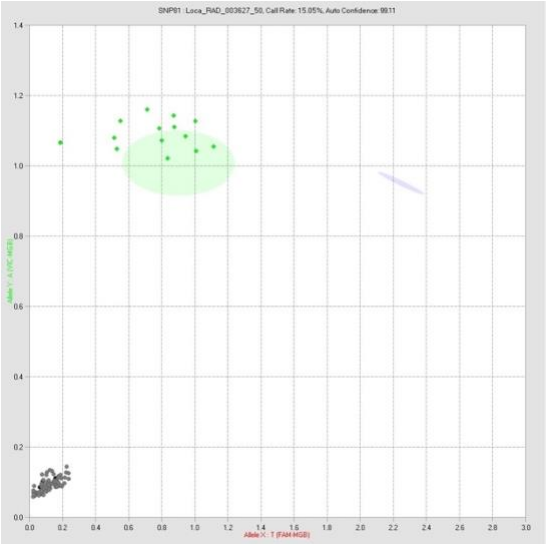
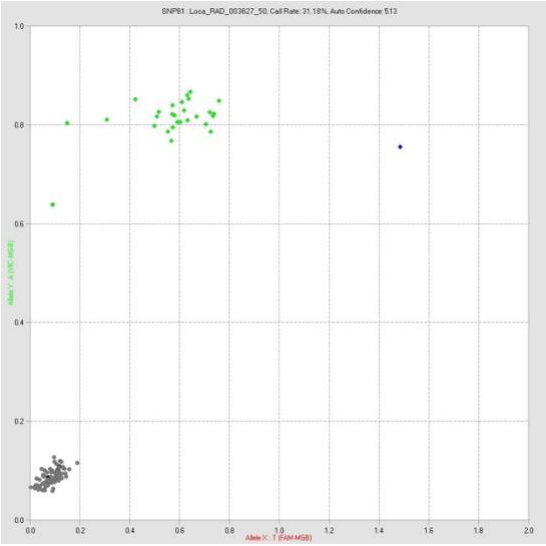
3 groupings? strange distribution. Kind of a mess. FAM homozygotes smear towards the NTCs making it difficult to tell where they stop and the failures start and the other homozygotes smear towards the heterozygotes, making it difficult to judge where one cluster ends and the other begins.
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP35	Loca_RAD_009295_50

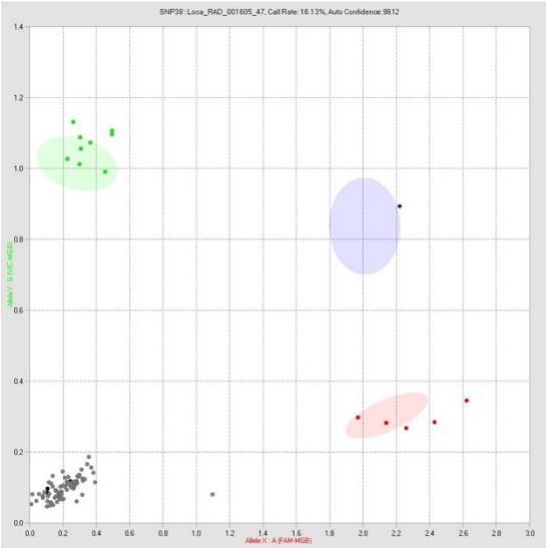
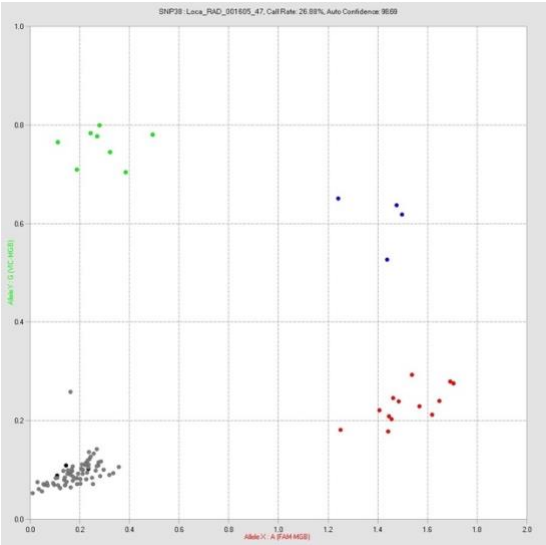
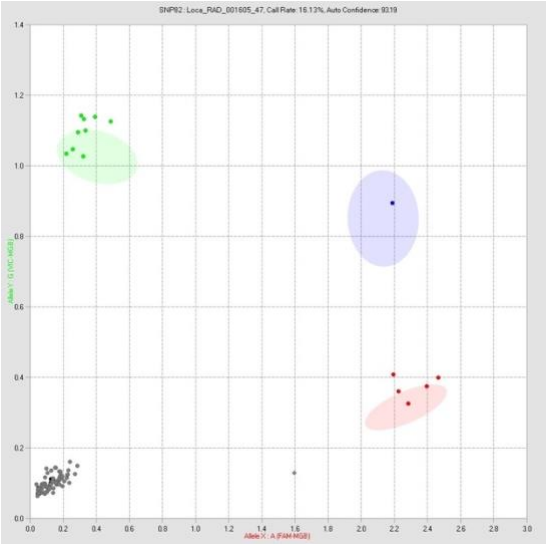
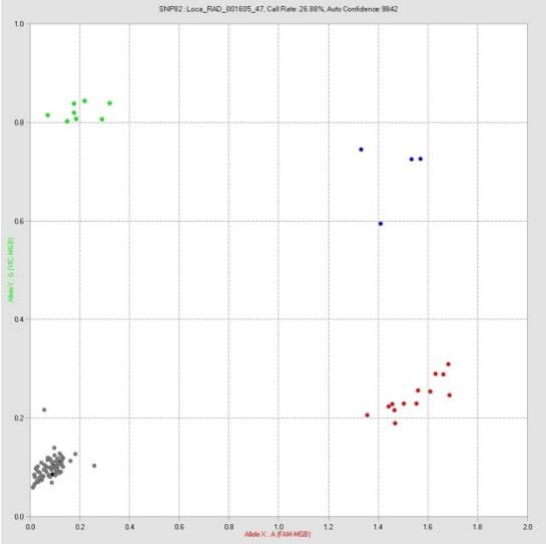
some samples fail, but 3 distinct groupings
category green (ok to use)
1 replicate mismatch (sample 226)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP36	Loca_RAD_009389_64

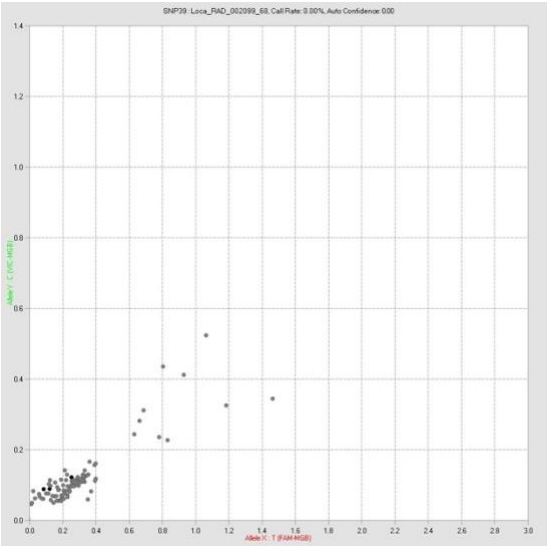
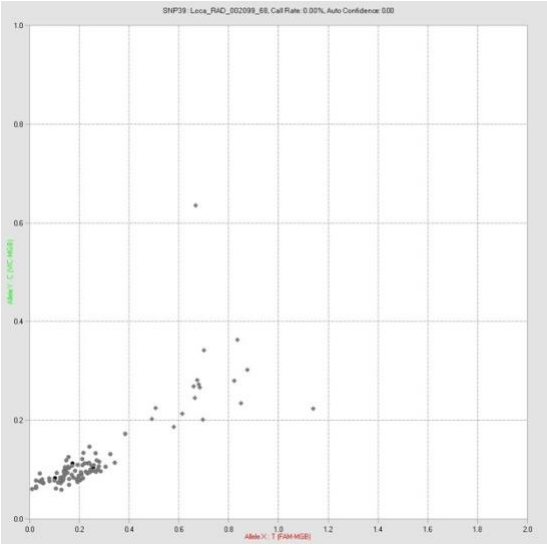
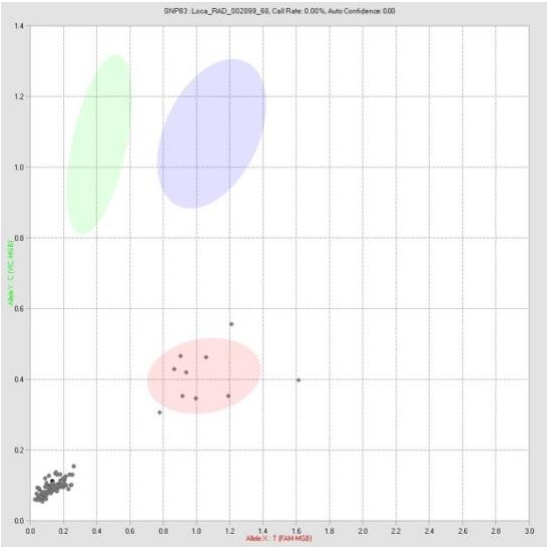
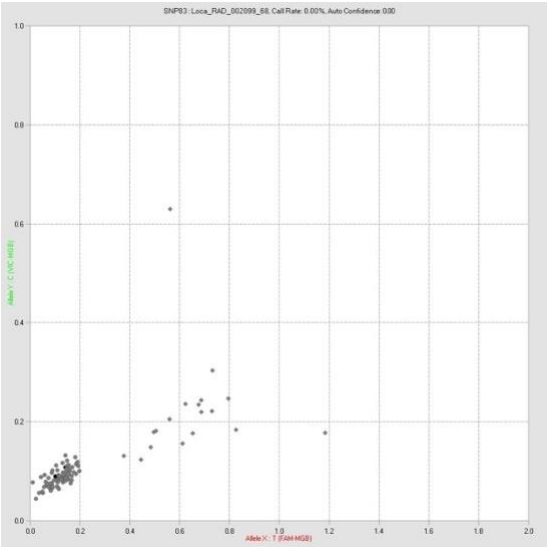
appears fixed, some outliers
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP37	Loca_RAD_003627_50

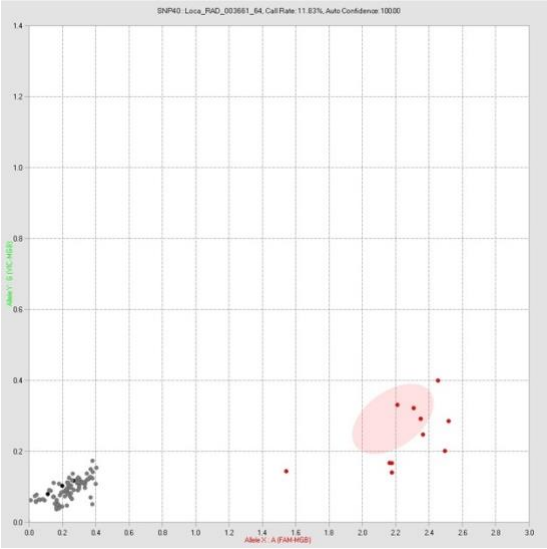
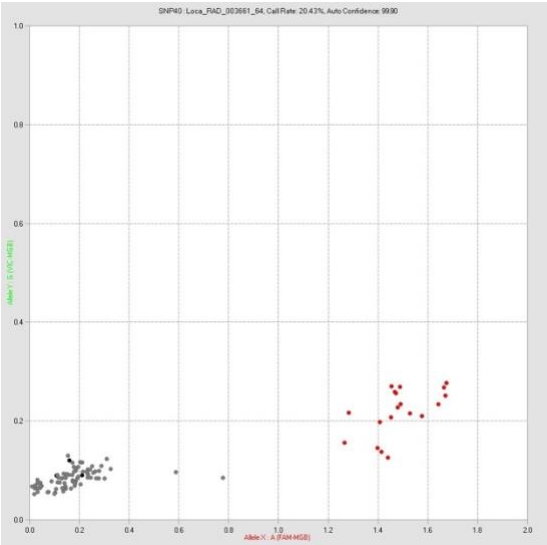
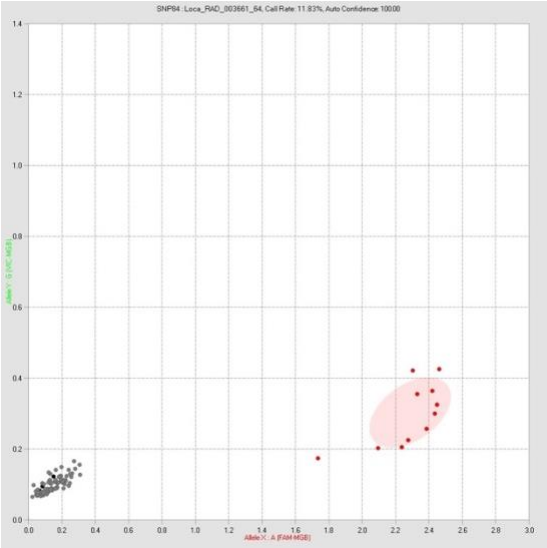
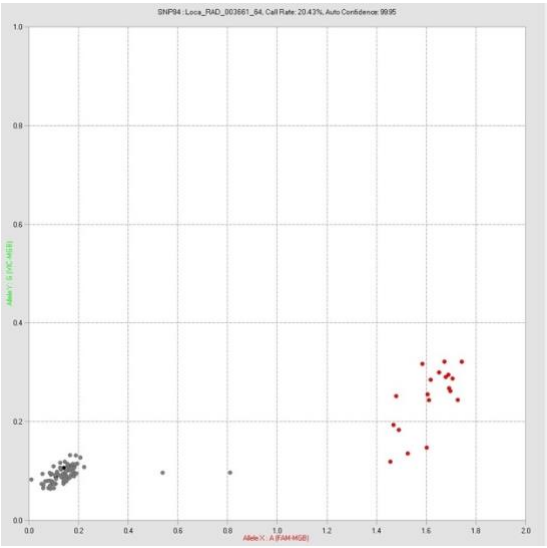
appears fixed, some outliers
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP38	Loca_RAD_001605_47

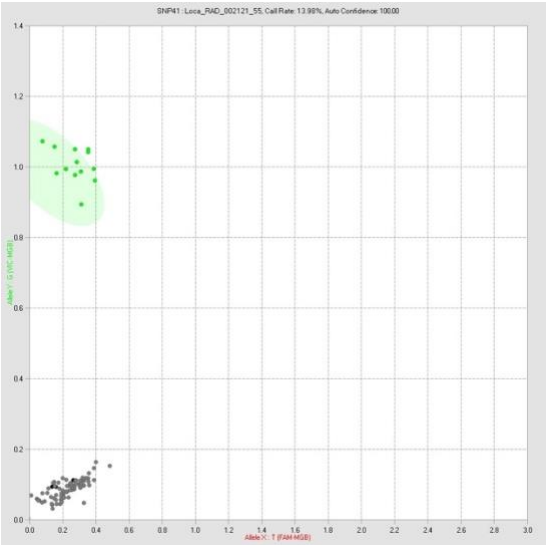
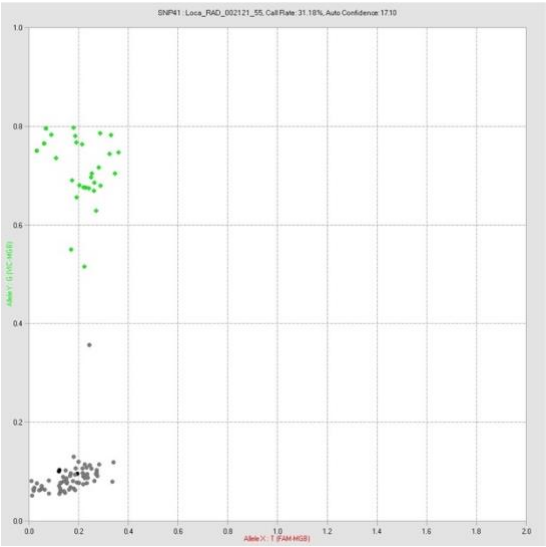
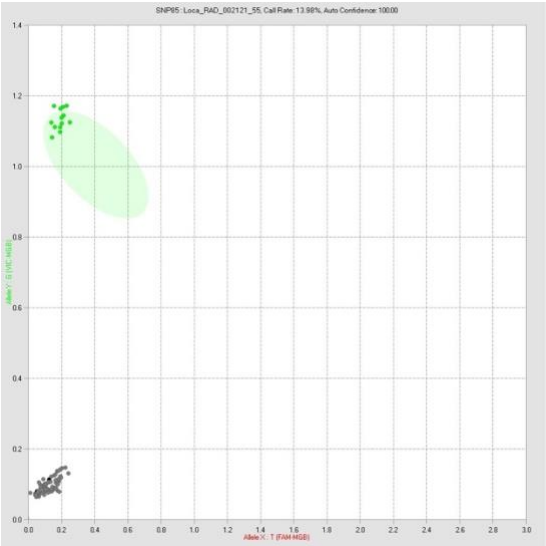
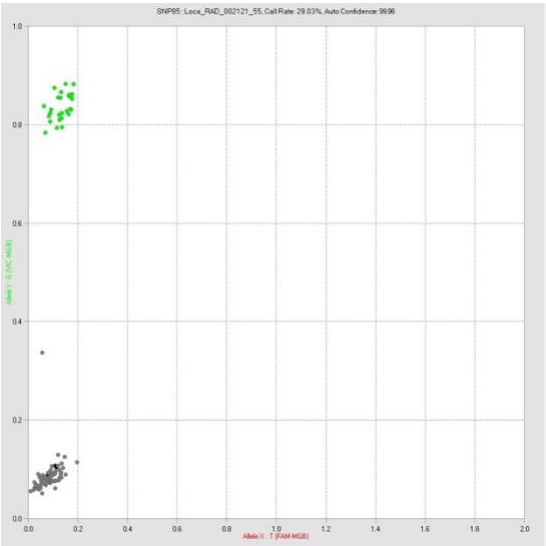
3 clearly distinct groupings
category green (ok to use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP39	Loca_RAD_002099_68

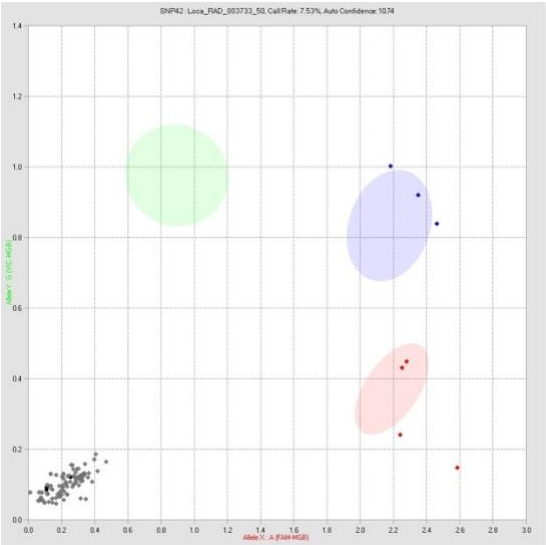
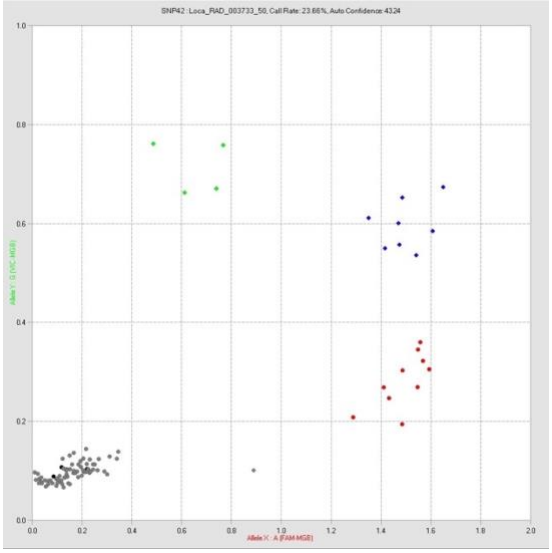
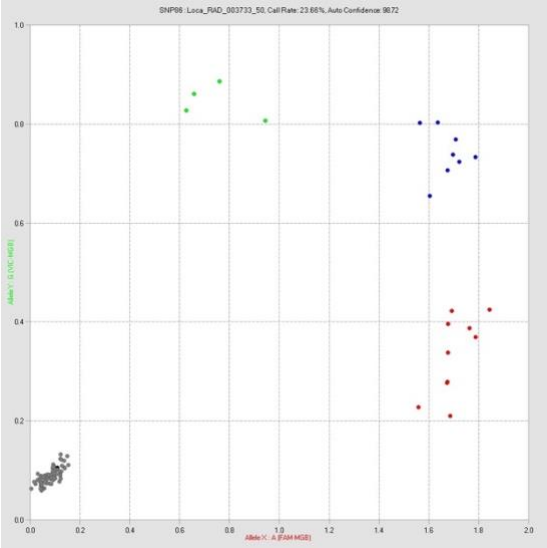
appears fixed, some outliers. This assay is just a mess with these samples, looks like some things are amplifying but really hard to judge what cluster they might be.
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP40	Loca_RAD_003661_64

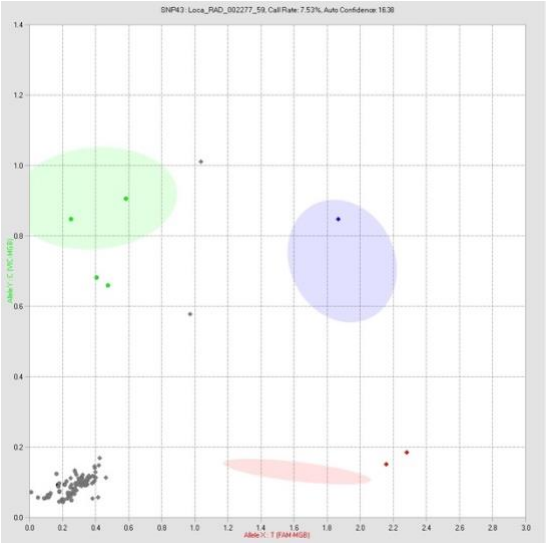
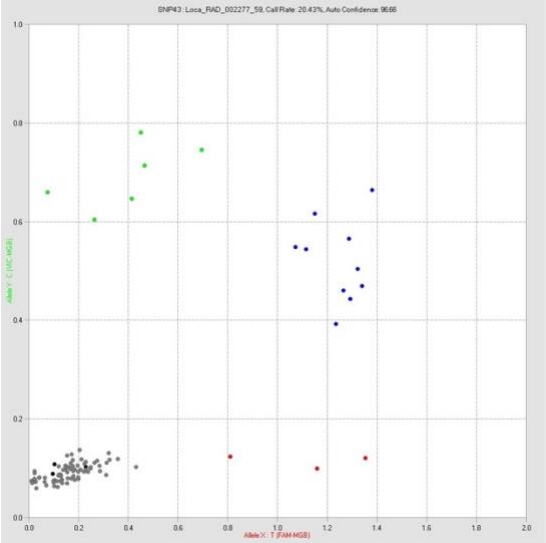
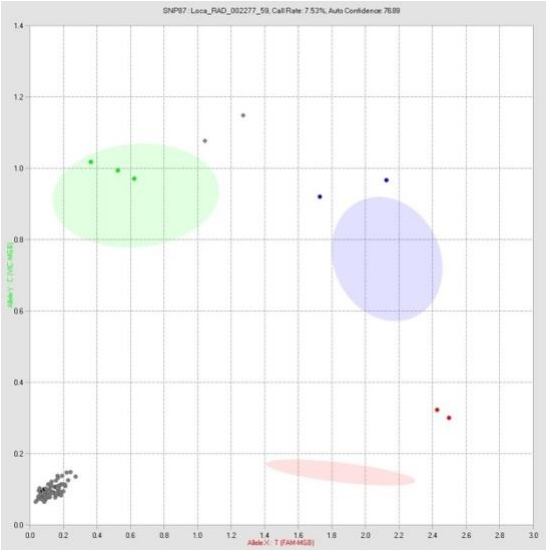
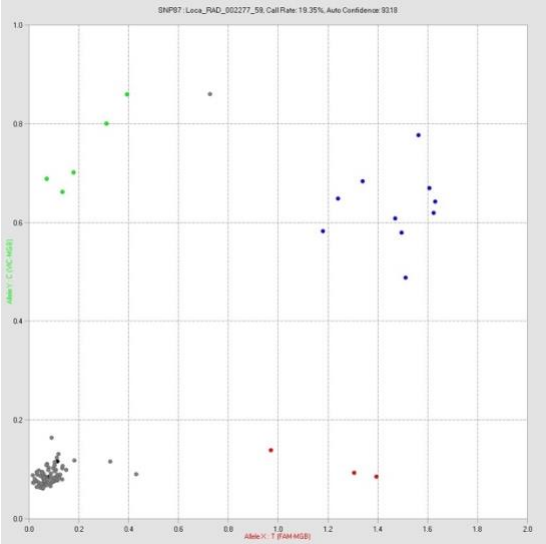
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP41	Loca_RAD_002121_55

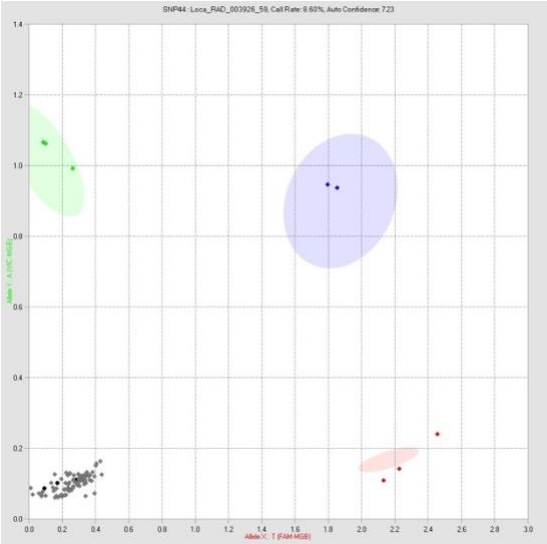
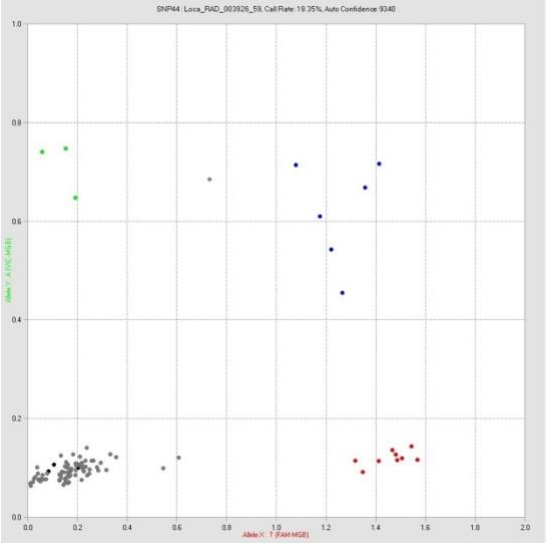
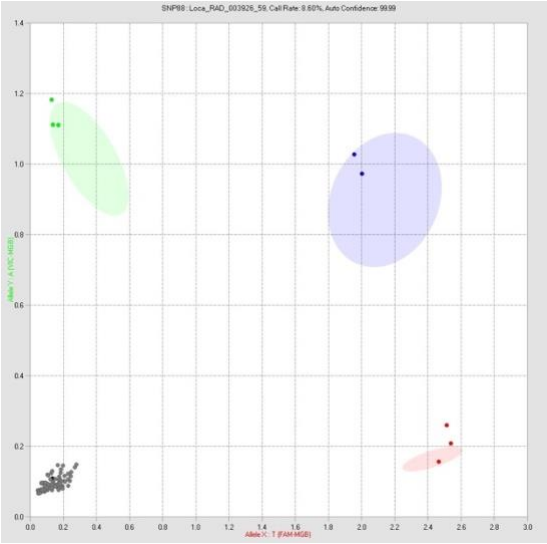
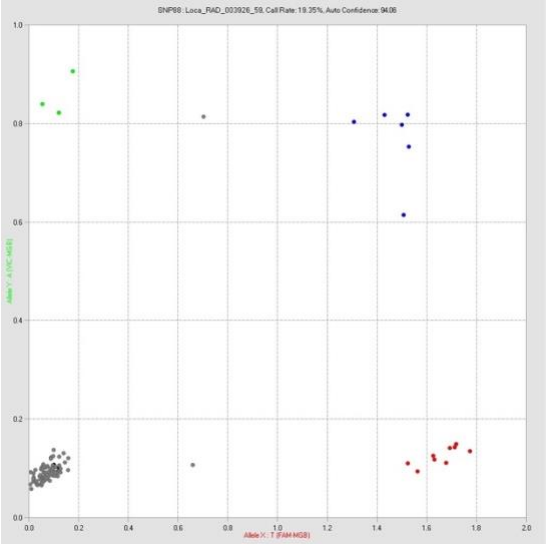
appears fixed, some outliers
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP42	Loca_RAD_003733_50

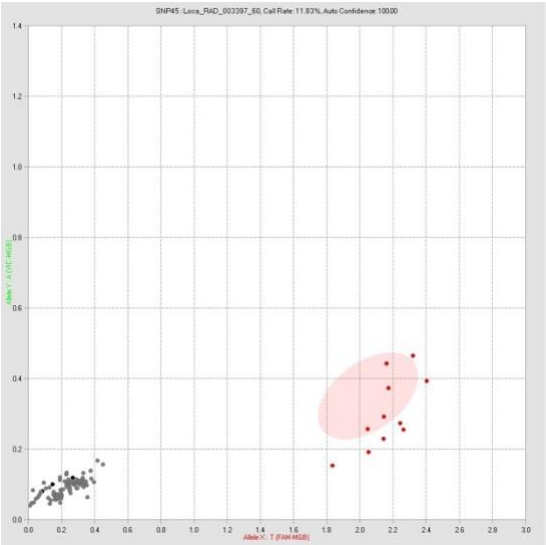
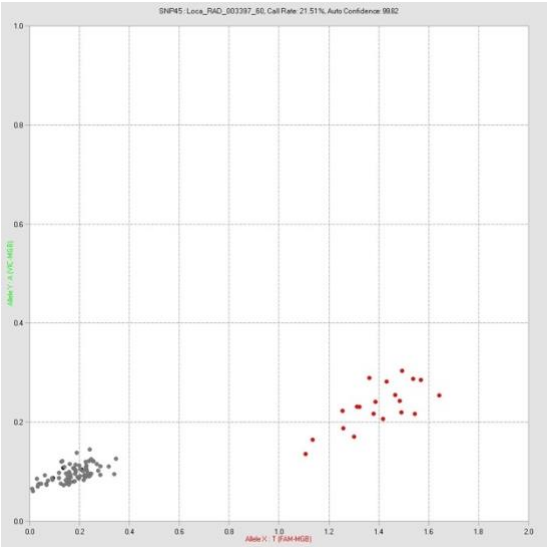
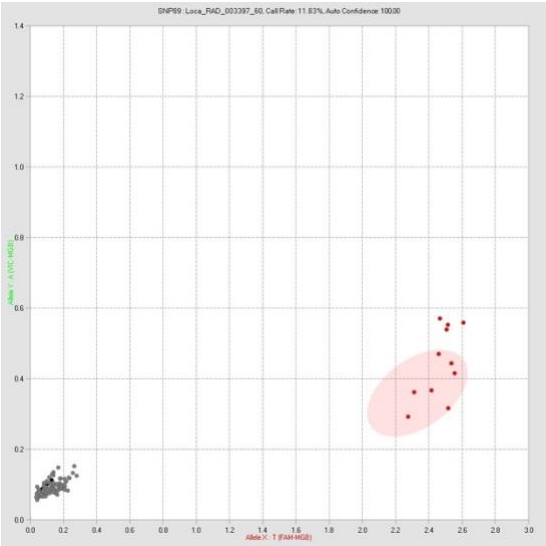
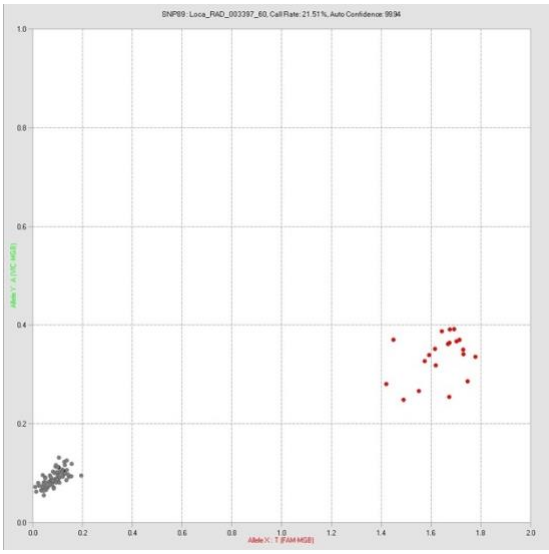
3 clearly distinct groupings
category green (ok to use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP43	Loca_RAD_002277_59

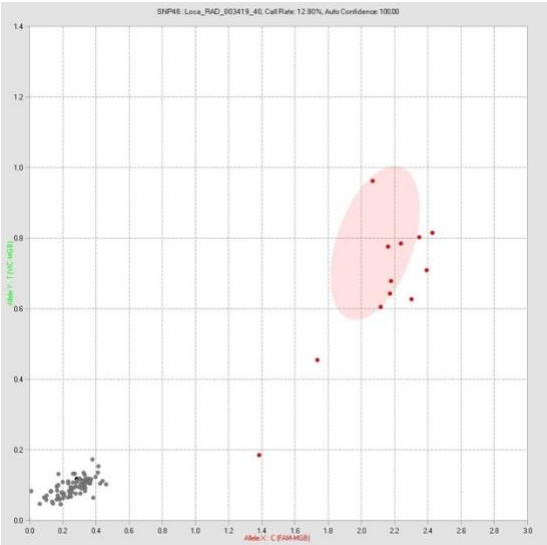
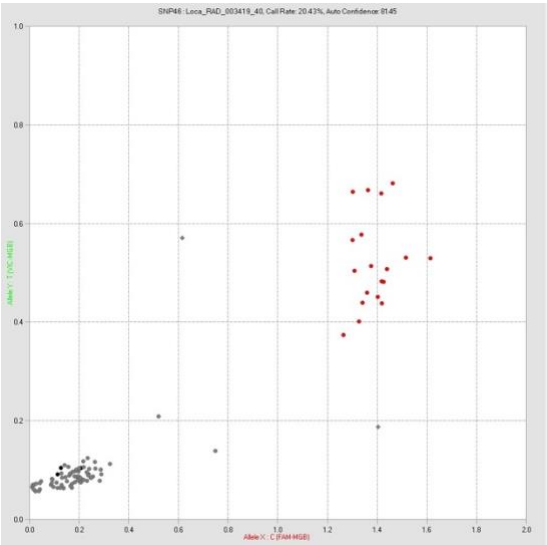
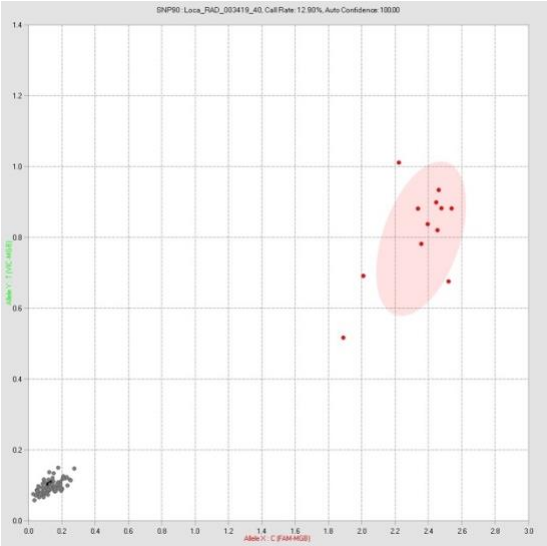
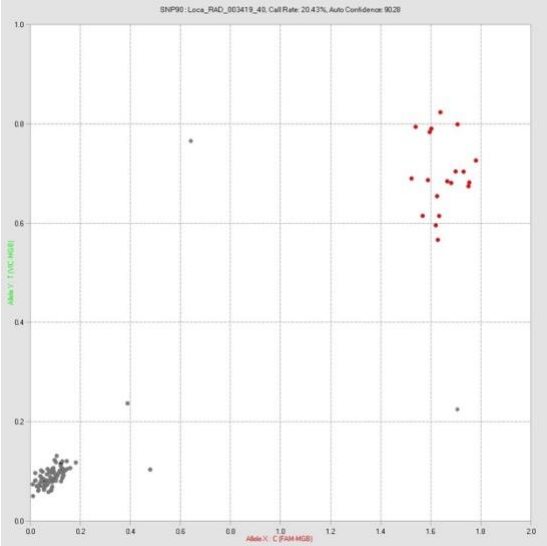
some samples fail, but 3 distinct groupings. Clusters a little loose, but had good amount of space between them on these chips, but I'd keep an eye on it—they might pull towards each other if more samples amplify, making it difficult to score.
category yellow (use with caution)
3 replicate mismatches (samples 14, 23, 170)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP44	Loca_RAD_003926_59

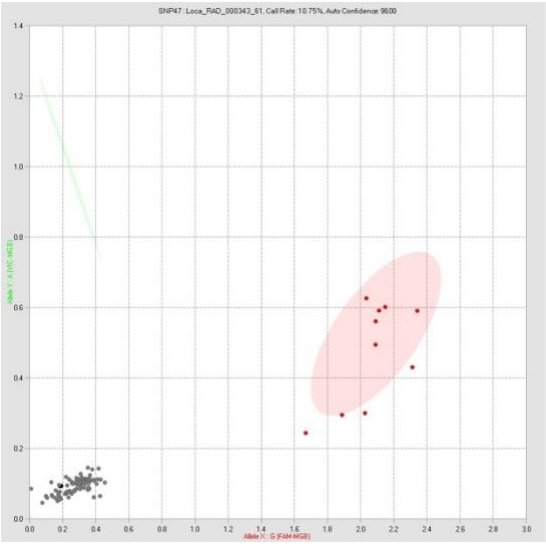
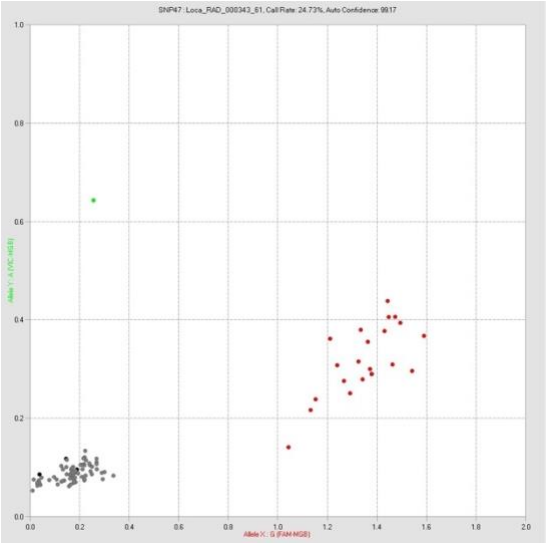
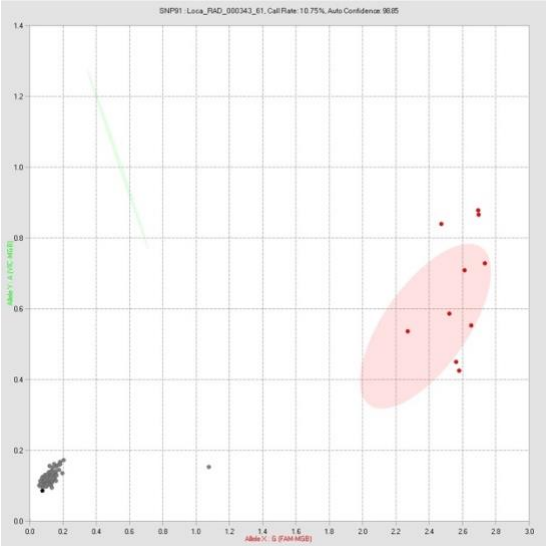
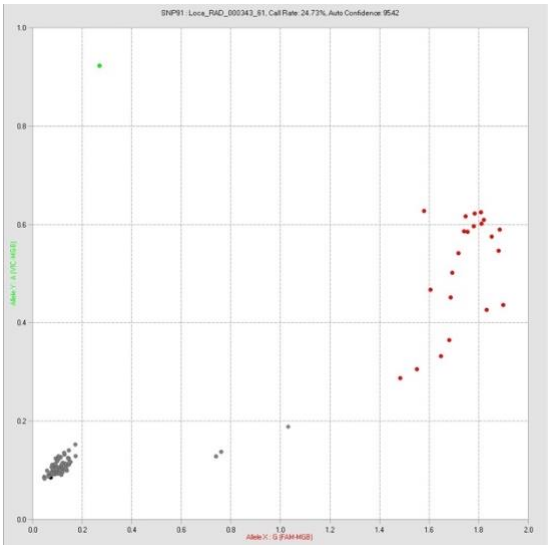
some samples fail, but 3 distinct groupings
category green (ok to use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP45	Loca_RAD_003397_60

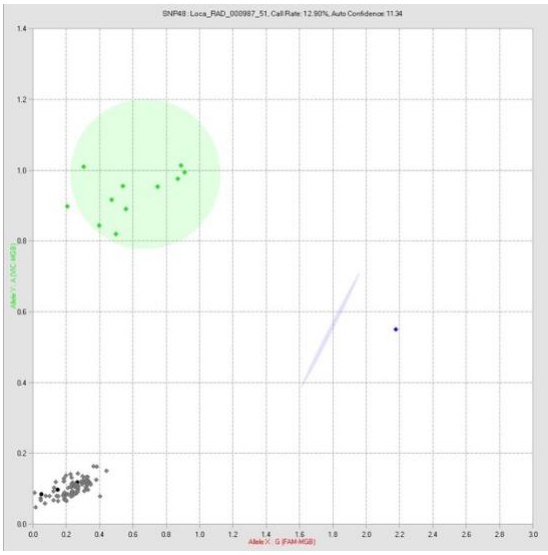
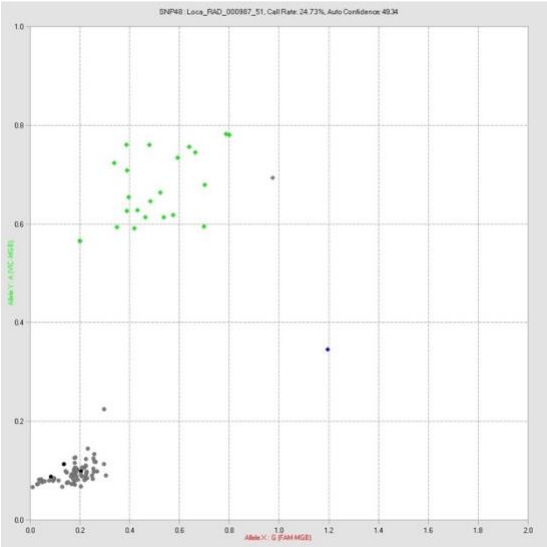
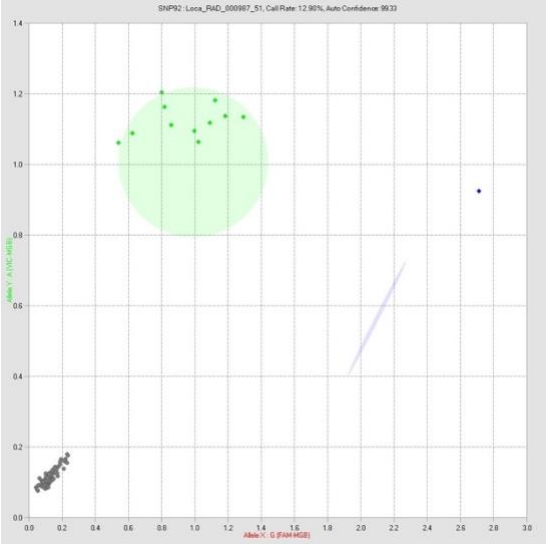
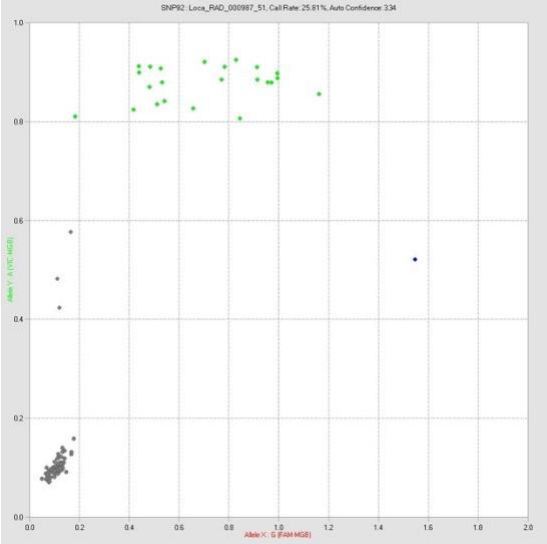
appears fixed
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP46	Loca_RAD_003419_40

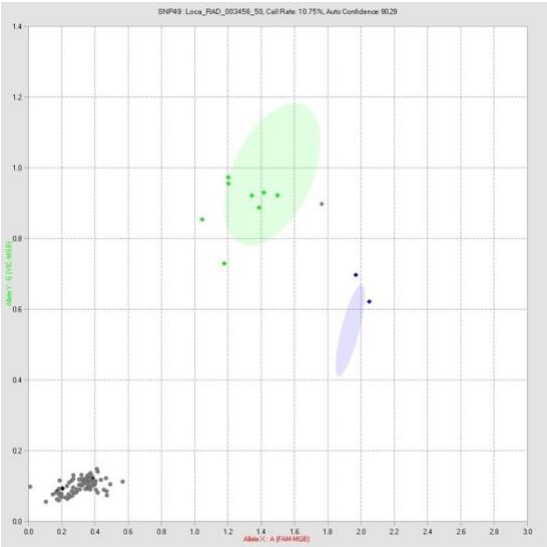
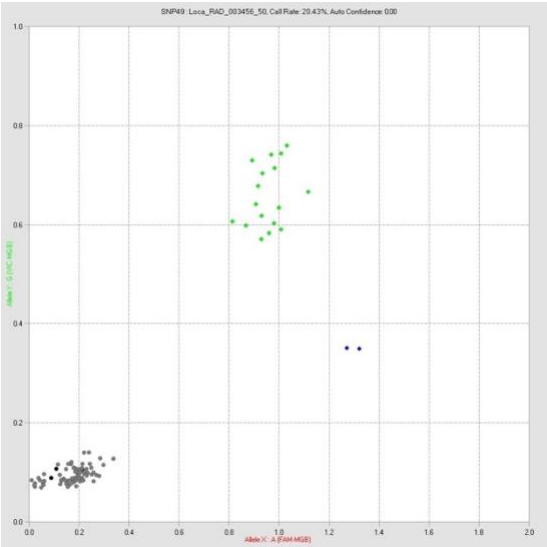
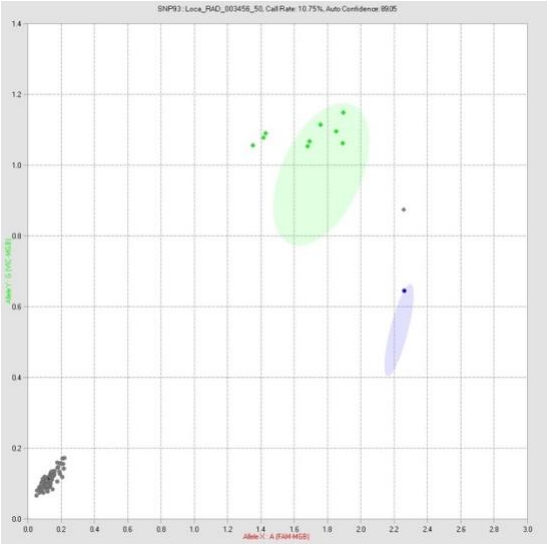
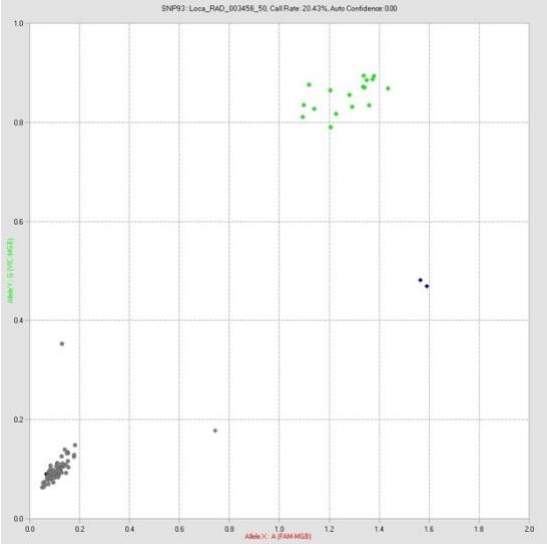
appears fixed, some outliers
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP47	Loca_RAD_000343_61

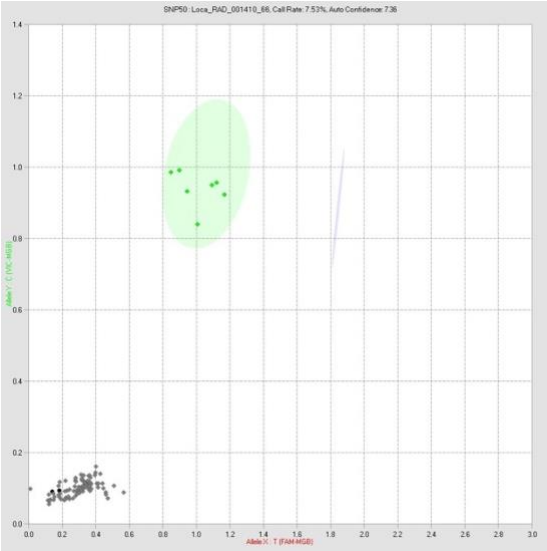
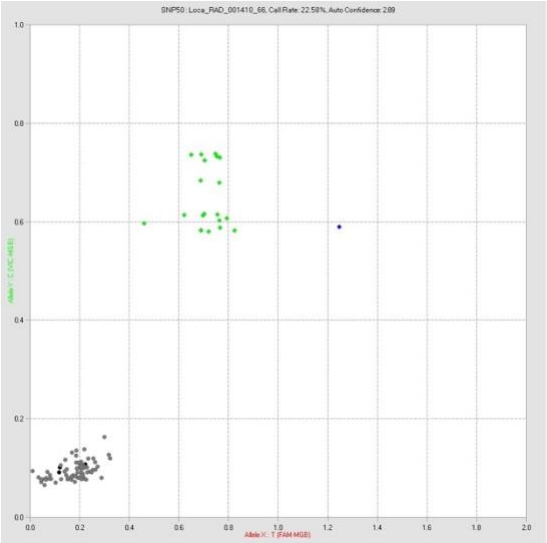
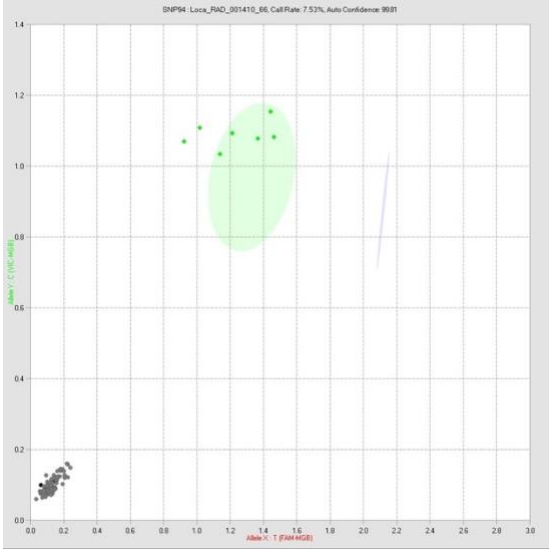
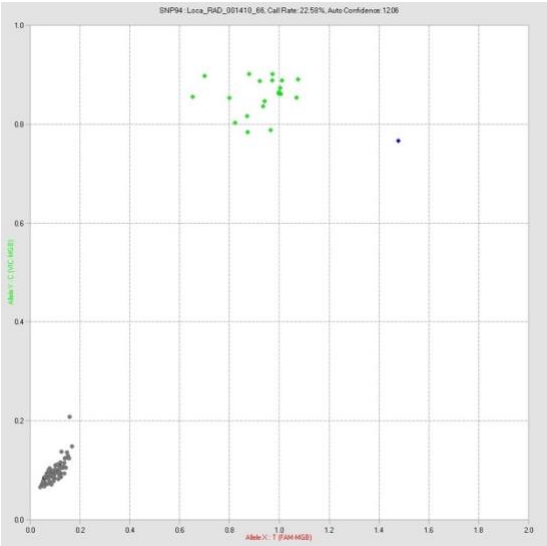
appears fixed, some outliers
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP48	Loca_RAD_000987_51

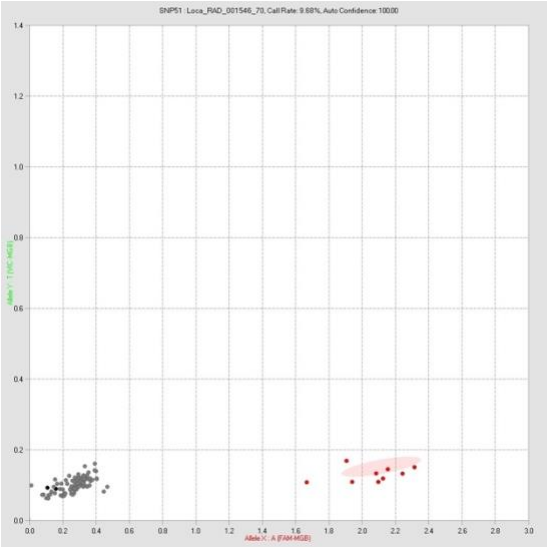
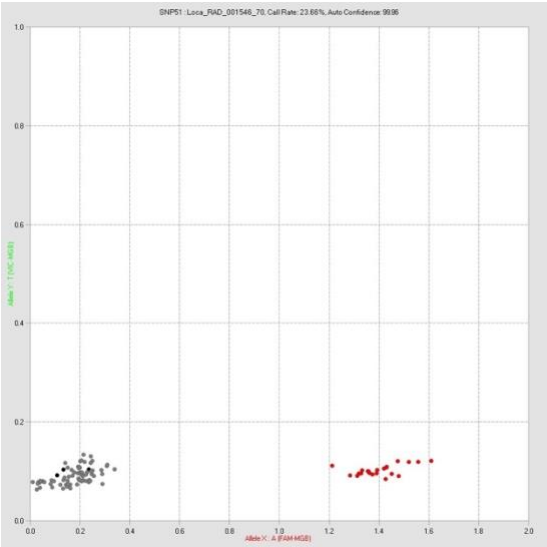
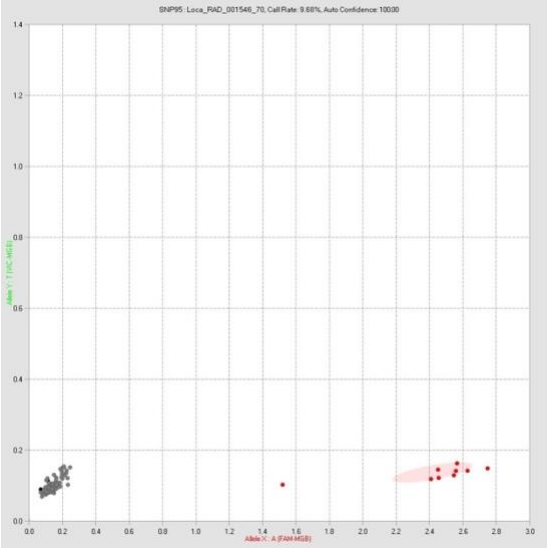
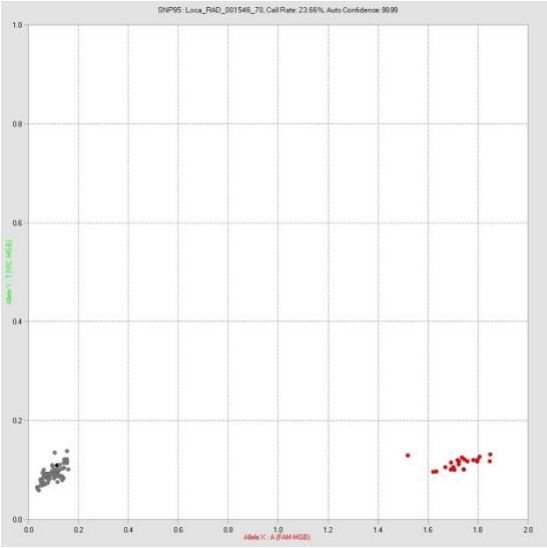
appears fixed, some outliers
category gray (do not use)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP49	Loca_RAD_003456_50

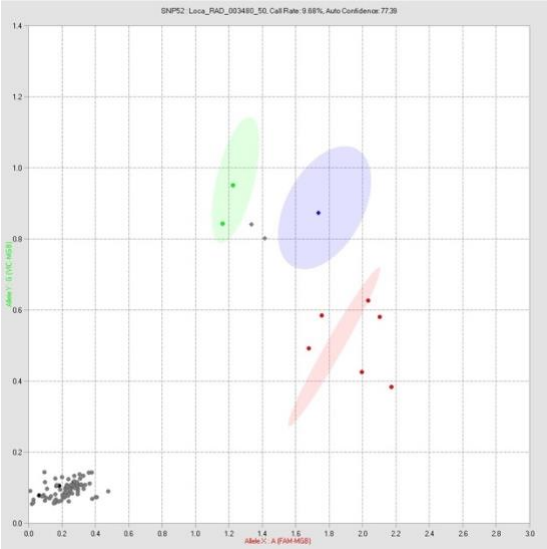
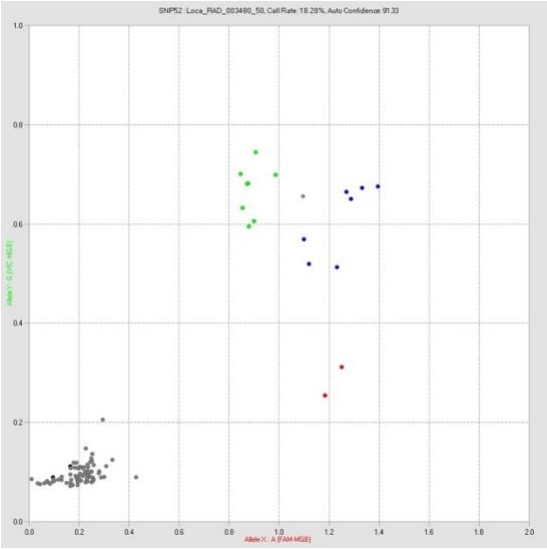
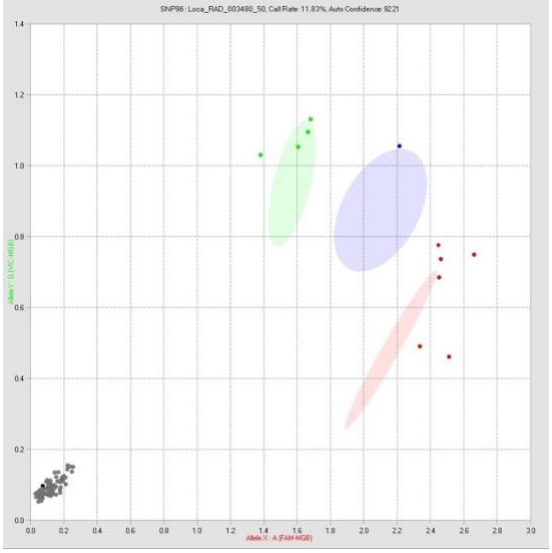
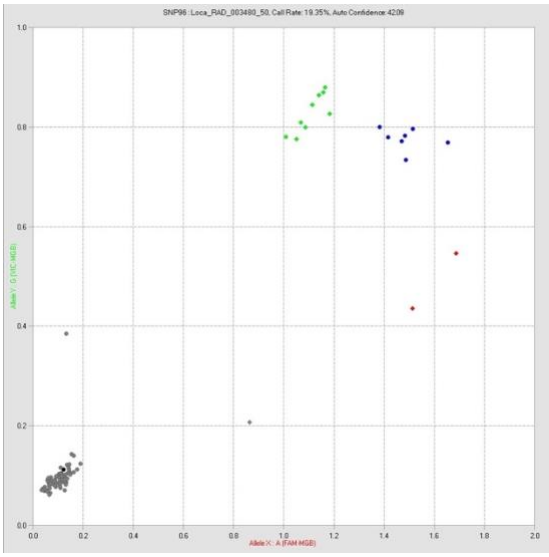
strange distribution, only two groupings? HEX (VIC on axes) homozygote amplifying closer to where you'd expect to see a heterozygote cluster, but based on HW equilibrium expectations it must be the heterozygote cluster.
category yellow (use with caution)
2 replicate mismatches (samples 14, 23)

Replicates on Chip 1	Replicates on Chip 2
	
	
SNP50	Loca_RAD_001410_66

appears fixed, some outliers
category gray (do not use)

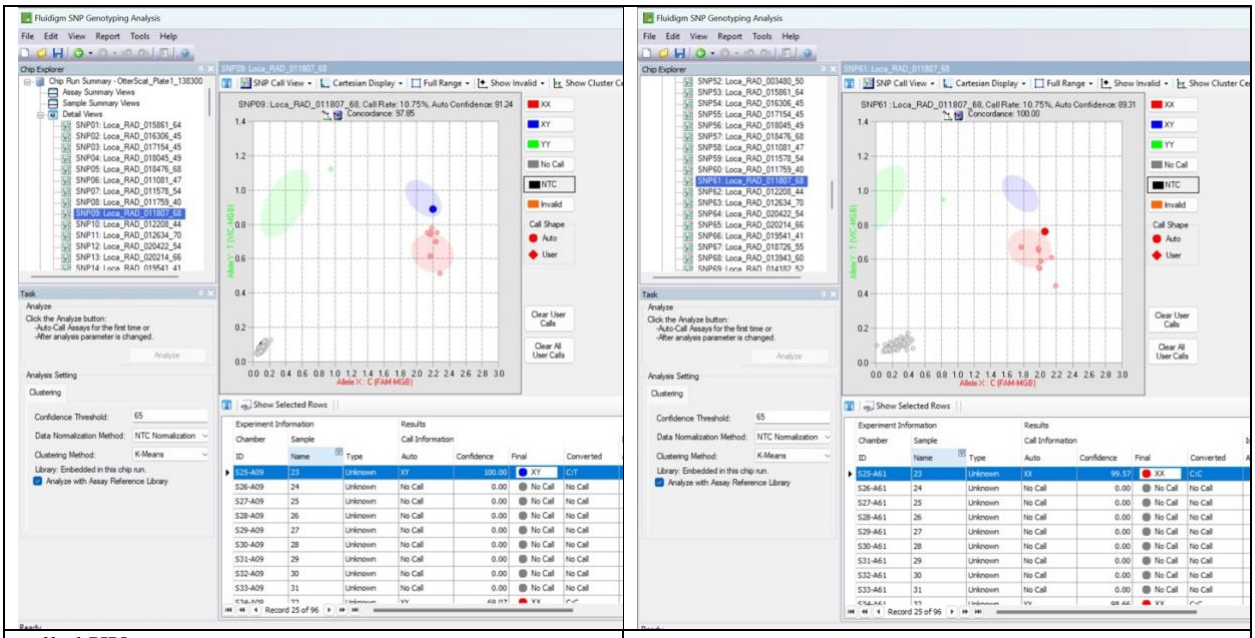
Replicates on Chip 1	Replicates on Chip 2
	
	
SNP51	Loca_RAD_001546_70

appears fixed, some outliers
category gray (do not use)

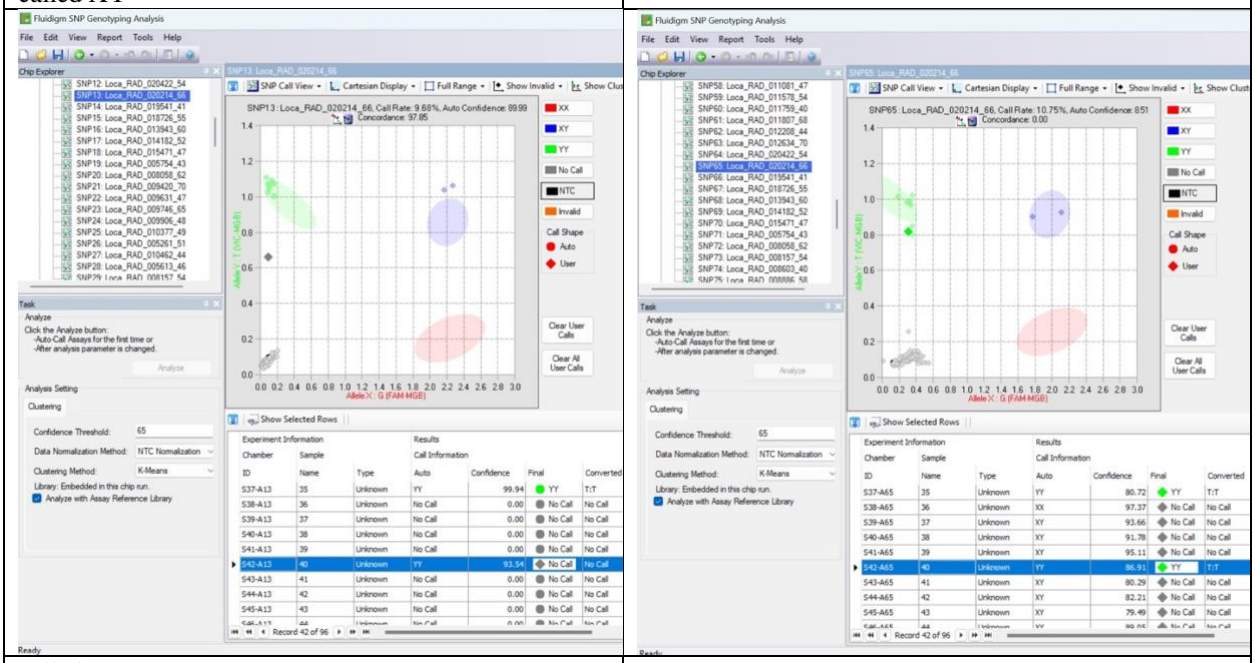
Replicates on Chip 1	Replicates on Chip 2
	
	
SNP52	Loca_RAD_003480_50

3 groupings? strange distribution. These clusters all amplify really close. Seem to be enough space to score them on chip 2, but this assay would be very difficult to score if not all three clusters were observed.
category yellow (use with caution)
2 replicate mismatches (sample 14, 119)

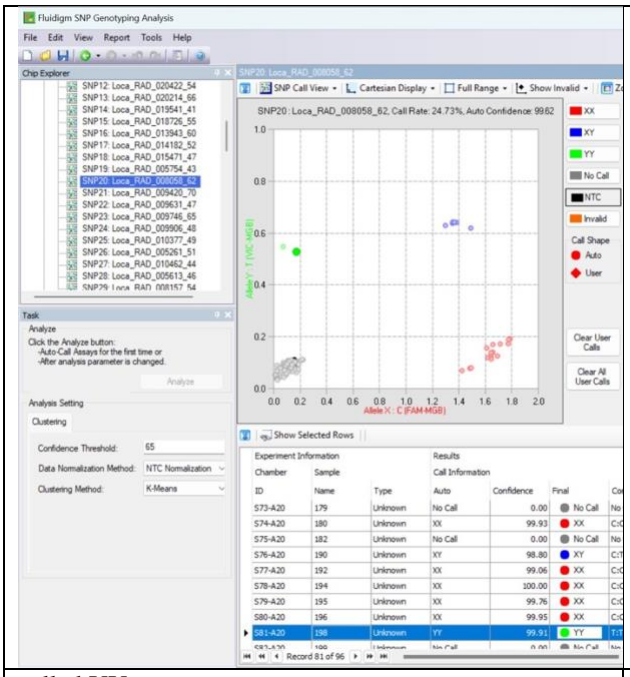
Appendix 2. Fluorescence plots and corrected allele calls for 11 individual sample SNP assays with mismatched replicate calls. The heavily colored dot in each plot is the sample in question.

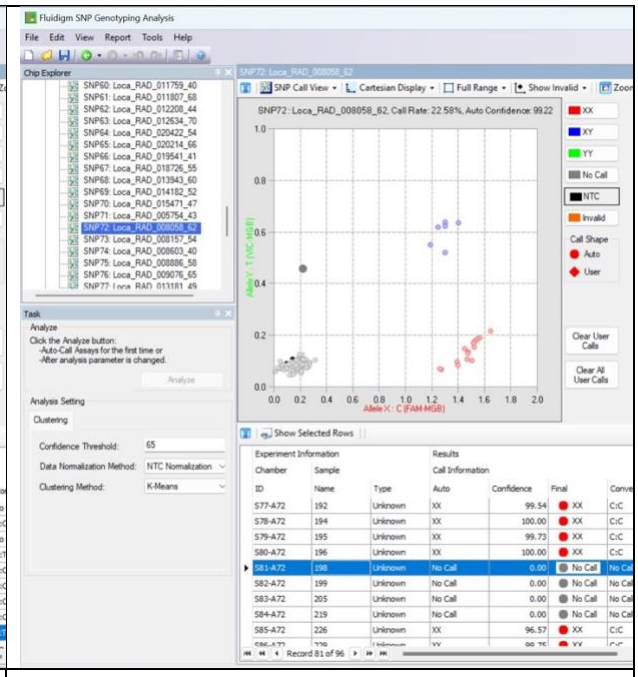


called XY

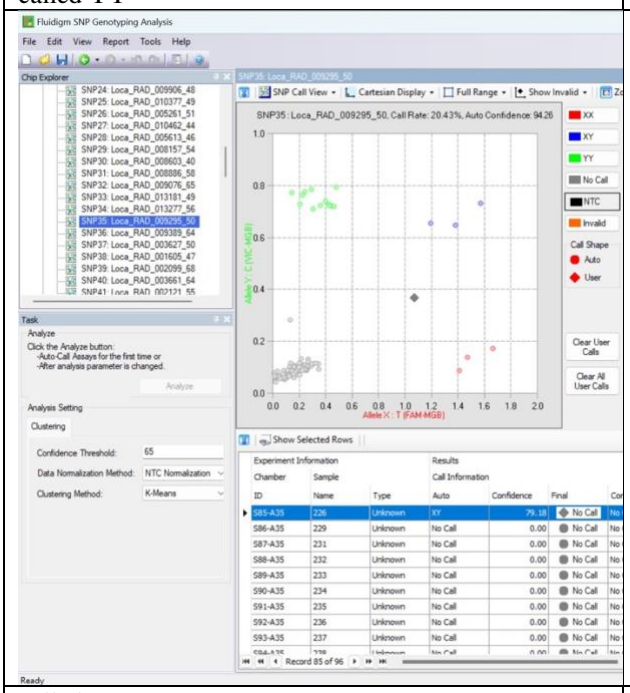


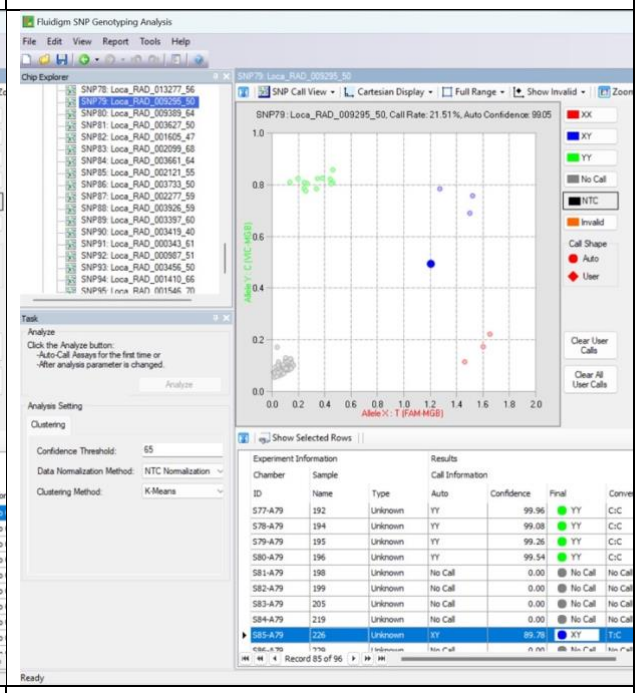
called YY



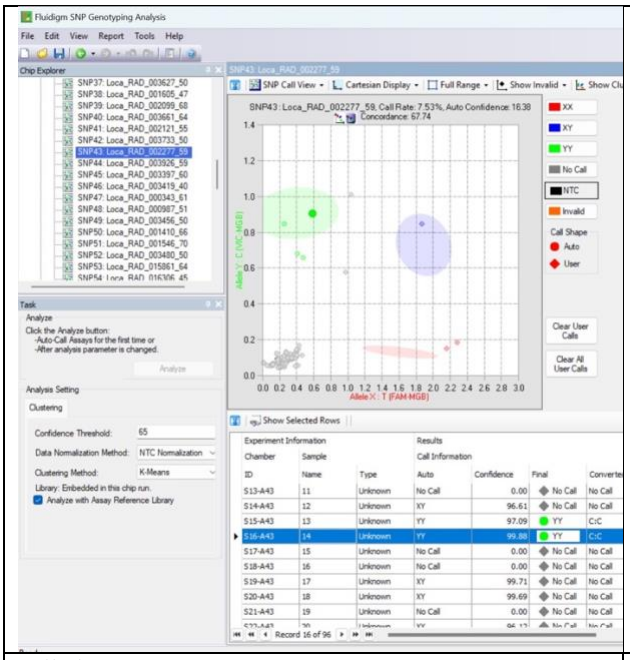


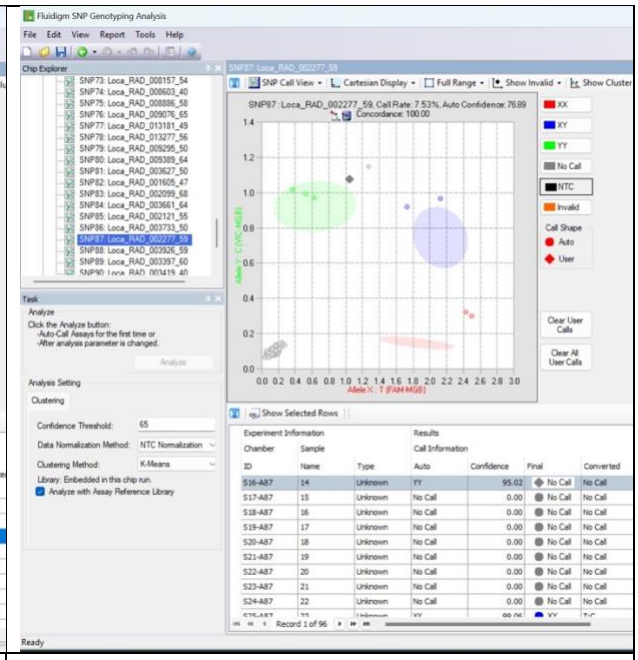
called YY



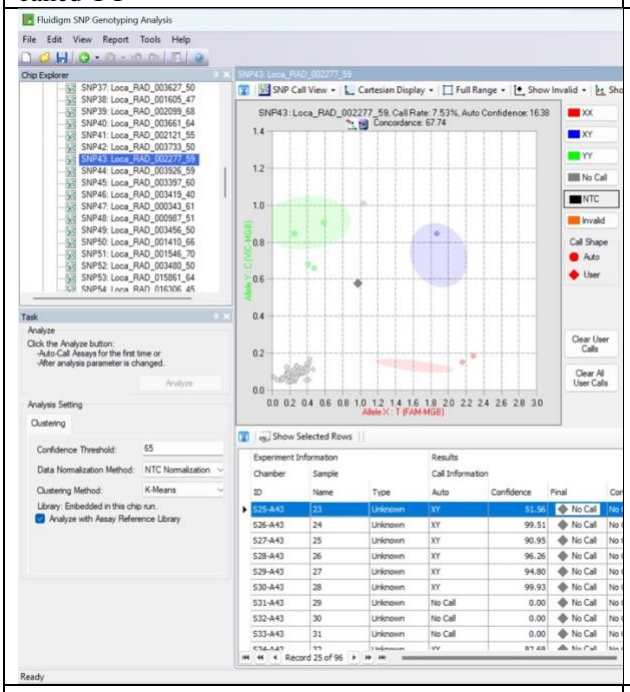


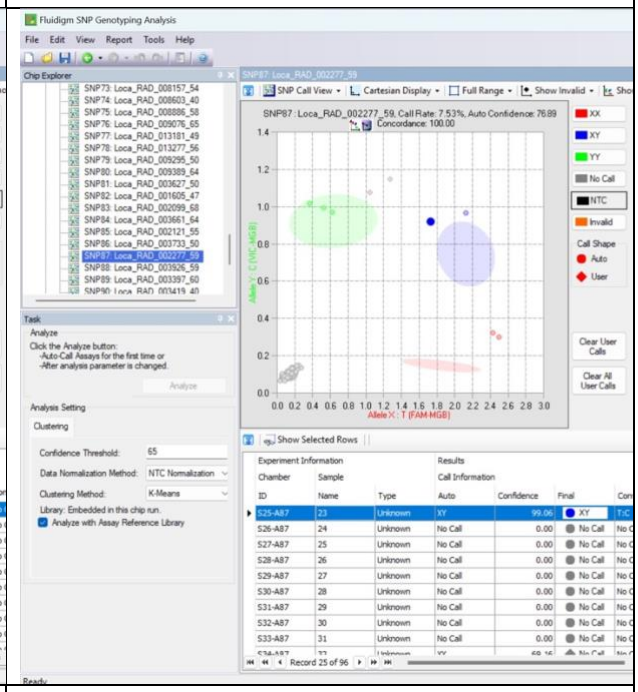
called XY



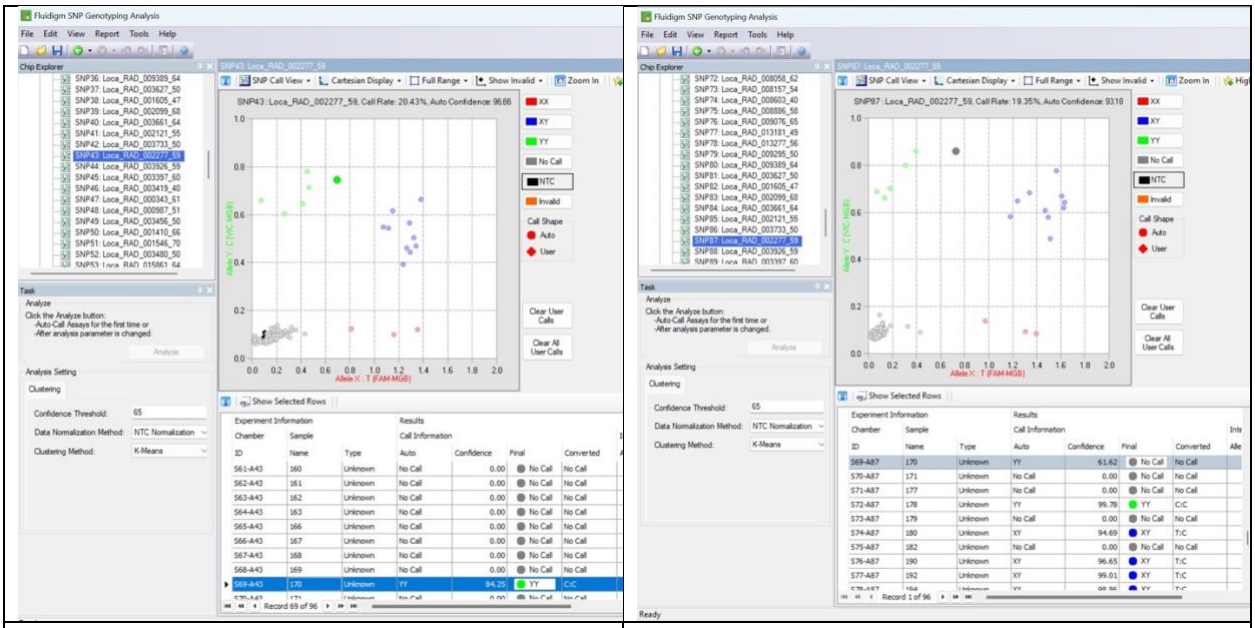


called YY

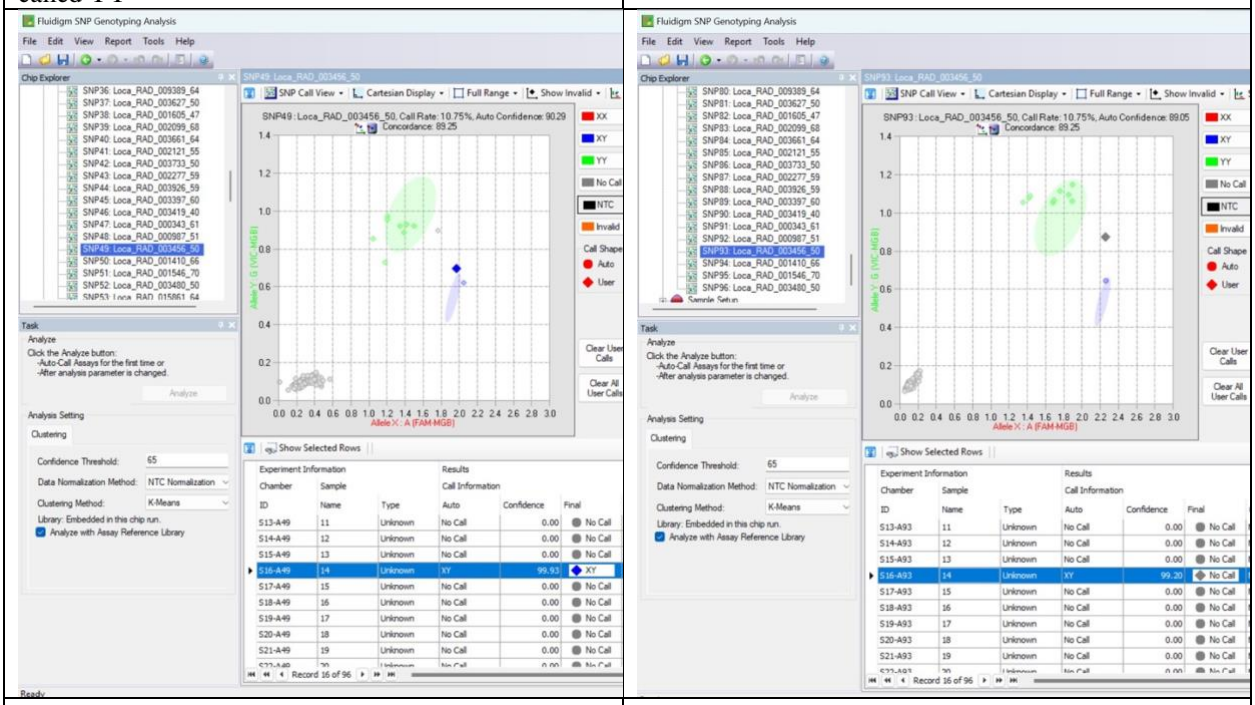




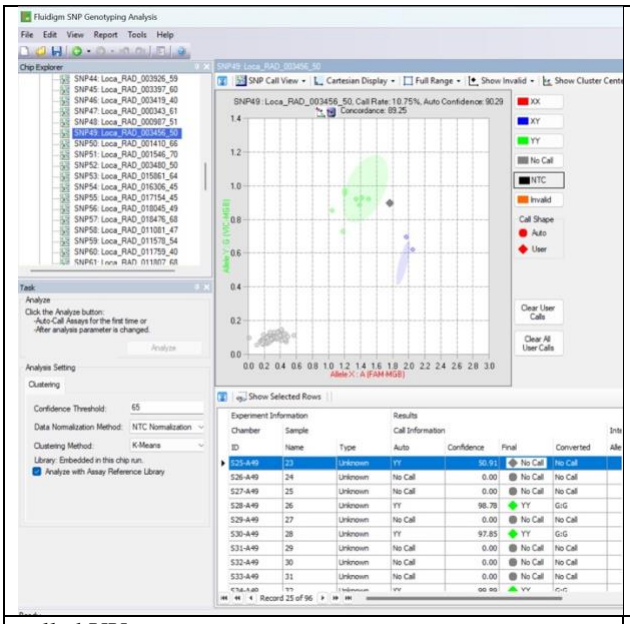
called XY

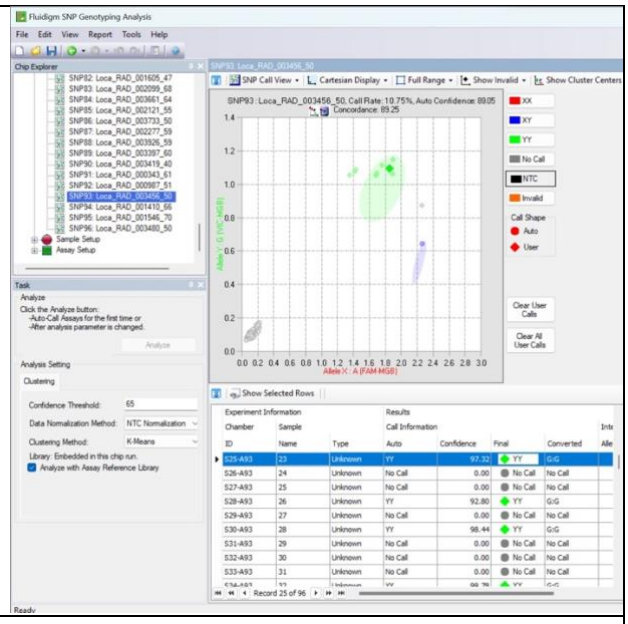


called YY

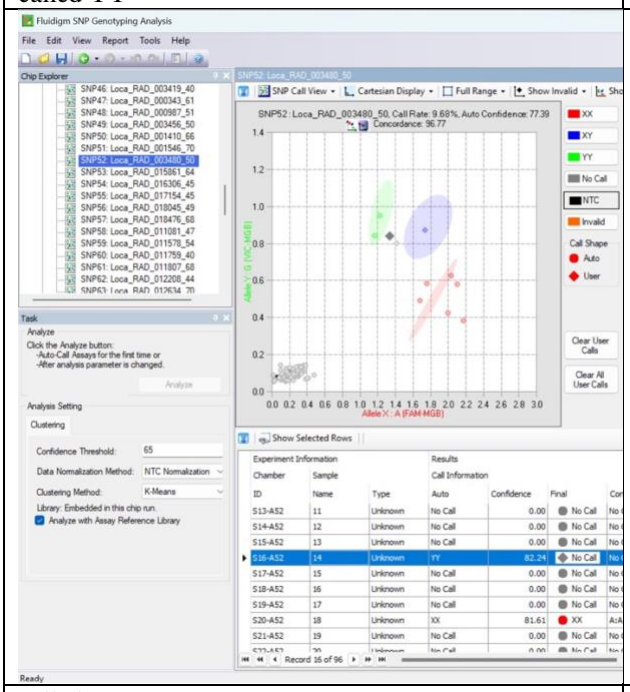


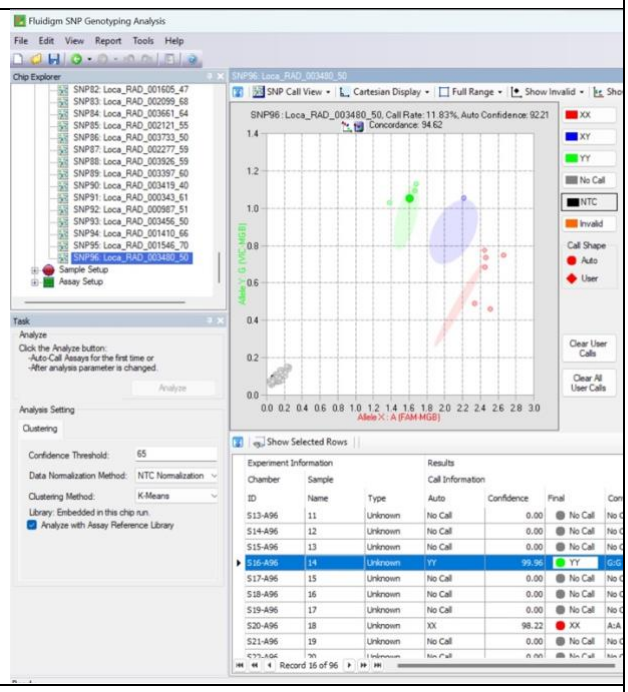
called XY



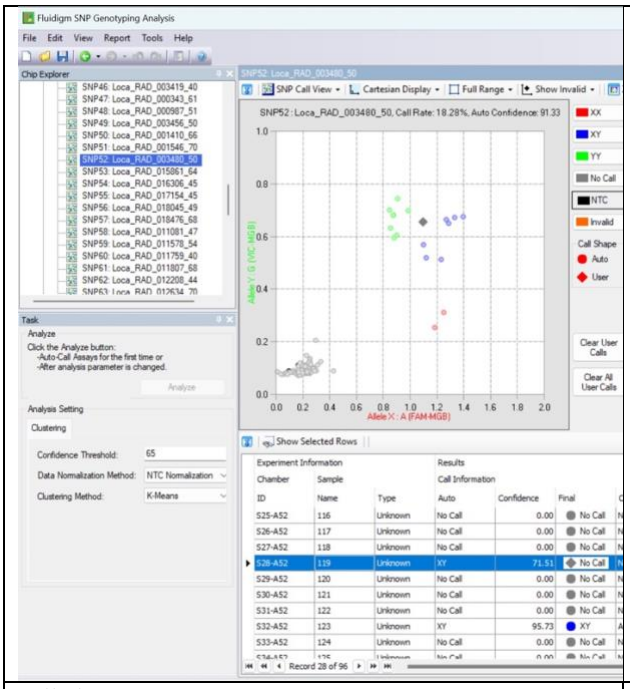


called YY

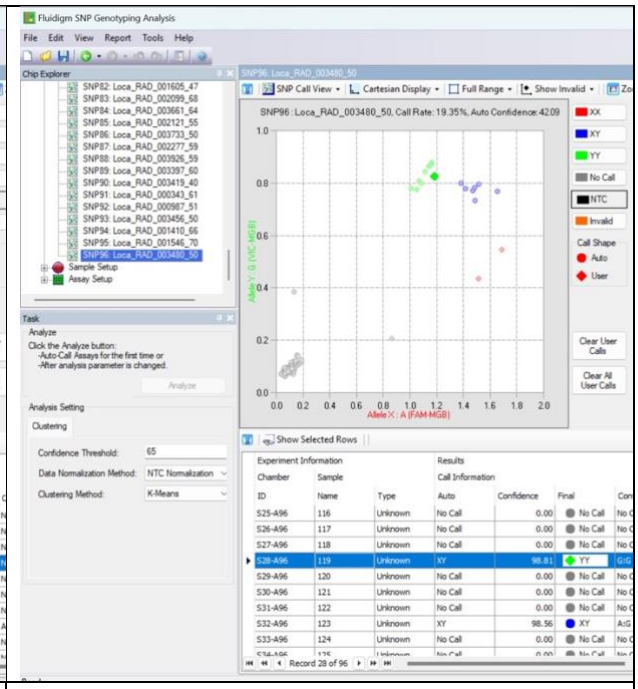




calledYY


Fluidigm SNP Genotyping Analysis
File Edit View Report Tools Help
Chip Explorer
SNP52: Loca_RAD_003480_50, Call Rate: 18.28%, Auto Confidence: 91.33
Allele Y : G (VIC-MGB)
Allele X : A (FAM-MGB)
Task
Analyze
Click the Analyze button:
-Auto Call Assays for the first time or
-After analysis parameter is changed.
Analysis Setting
Clustering
Confidence Threshold: 65
Data Normalization Method: NTC Normalization
Clustering Method: K-Means
Experiment Information
Results
Call Information
Record 28 of 96

called YY


Fluidigm SNP Genotyping Analysis
File Edit View Report Tools Help
Chip Explorer
SNP96: Loca_RAD_003480_50, Call Rate: 19.35%, Auto Confidence: 42.09
Allele Y : G (VIC-MGB)
Allele X : A (FAM-MGB)
Task
Analyze
Click the Analyze button:
-Auto Call Assays for the first time or
-After analysis parameter is changed.
Analysis Setting
Clustering
Confidence Threshold: 65
Data Normalization Method: NTC Normalization
Clustering Method: K-Means
Experiment Information
Results
Call Information
Record 28 of 96