

Single Nucleotide Polymorphism Genotyping: Why Use Real-Time PCR and Considerations For Facility Set Up

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Introduction

- Laboratory aims
- Evaluation criteria
- Pilot study results
- Problems encountered
- Consumables optimisation

Facility Requirements

- Remit: Provide DNA extraction & SNP typing service to clinicians with little or no background in genetic research and also provide service to University research groups involved in SNP analysis
- Identification of client requirements is the most important aspect of set up and will influence all downstream evaluations/decisions – provides basis for evaluation criteria
- Would have to provide advice on all aspects of process to clinicians and provide all lab work
- Minimal staff dependency, so most advantageous would be ‘plug and play’ system that requires minimal optimisation with easiest automation
- Instrument would need to be used intermittently and be capable of both high and low throughput without compromising assay cost

Evaluation Criteria and Weighting

- Each laboratory will have an individual combination of criteria and weighting depending on client basis
- Allows unbiased evaluation of instruments
- Used University Procurement Dept. & an open tender process to obtain a wide selection of responses from instrument manufacturers for individual SNP typing, SNP typing pooled DNA, LIMS, & robotics
- Whole Life Costing takes into account purchase price, consumable costs, additional equipment required and staffing requirements over instrument lifetime

SNP Typing Platform

Ease of use/user friendliness	4	4.0%	Quality (of output/accuracy etc)	15	15.0%
			Reliability	15	15.0%
			Footprint	5	5.0%
Consumables costs (see model)	30	30.0%			
Impact Factor	5	5.0%			
Additional hardware to operate	5	5.0%			
Compatability with existing platforms	3	3.0%			
Integrated solution	10	10.0%			
Additional applications	8	8.0%			

Type of detection		5		5.0%
Type of chemistry		5		5.0%
Throughput of platform (SNPs/day)	10,000	25	x	25.0%
Range of detection (input PCR volume)	0.01 - 5 ul	10		10.0%
"Multiplexability" of reaction	1 to 20	7		7.0%
Resolution of System		8		8.0%
Software/LIMS compatibility	yes	20	x	20.0%
Ability to automate/robot compatibility	yes	20	x	20.0%

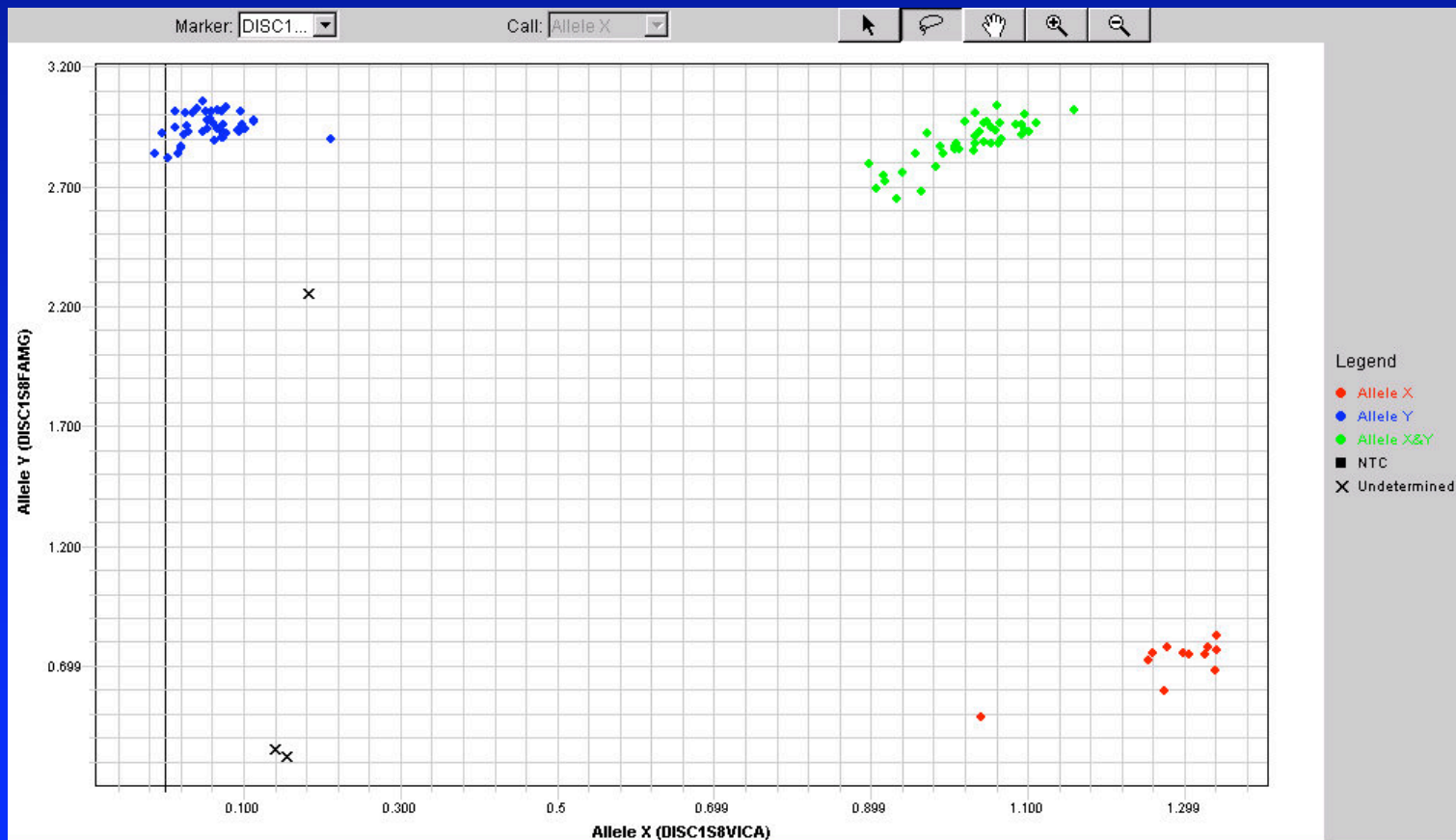
Decision to Purchase

- Choice between AB 7900HT & Sequenom MassArray
- Chose 7900HT as SNP typing platform
- Scored well on
 - Cost of analysis
 - Throughput
 - Ease of use ('plug and play', closed tube reaction)
 - Minimal staff requirement
 - Pre-formatted assays, both off-the-shelf and custom synthesis easy to use
- Also purchased
 - Dual arm Biomek FX, v quick set-up, correct volume range, disposable tips, other applications
 - Cytofluor plate reader

AB 7900HT

- Uses FAM & VIC labelled probes to provide discrimination between SNP alleles, incorporation of MGB & NFQ in probes provides good specificity – delivered in 1 tube format
- Simple set-up (genomic DNA + probes/primers + master mix), readily automatable using FX at 5 μ l total assay volume using 384 well plates
- Closed tube reaction - no downstream processing of samples required - once read plate can be thrown away, can use 'off-line' PCR for greater capacity
- Data points plotted on simple graph of FAM vs. VIC fluorescence
- Simple interpretation of results

SNP plot example



Pilot Study Outline

- The pilot was split into 2 parts
- 1st part compared results of potential customers in-house genotyping (SNaPshot) to 7900HT typing (pre purchase)
- 2nd part utilised AB's Assays-on-Demand and Assays-by-Design systems with in-house automation to explore our sample processing pipeline (post purchase)

Pilot Study: part 1

- 480 samples to be run in triplicate
- 5 SNPs to be assayed (pre-existing SNaPshot assays)
- DNA prep from a variety of sources, normalised using UV spec
- SNaPshot genotyping performed on both AB 377 and 3700 by PIs, designed and optimised in-house
- Real-time PCR SNP genotyping assays developed via AB's Assays-by-Design (AbD) service

Pilot Study: part 1 summary

SNaPshot

- ~80% calls of all wells (1st pass), 2% disagreement in replicates

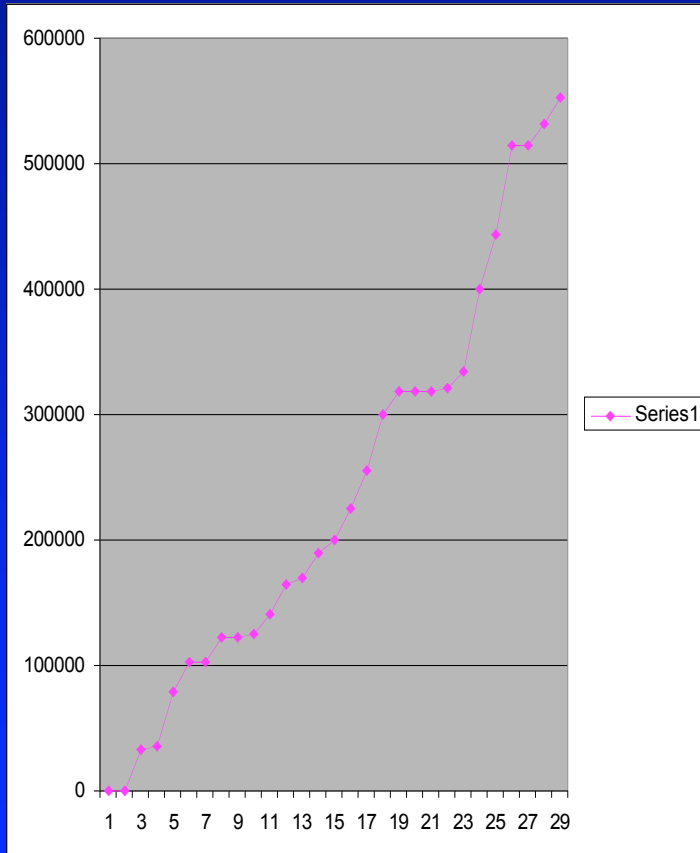
7900HT

- 1 assay failed functional testing using AbD service
- Called 99.7% of all wells (n=5,760, 19 no-calls) & 99.2% of all samples (4/480 samples not called)
- 100% agreement between replicates where a call was made
- All NTCs identified blind
- 2 disagreements between SNaPshot and 7900HT
- AbD service appeared to produce good, functional assays requiring no further in-house QC
- Re-submitted AbD failure with more surrounding sequence (~150bp) - assay now working.

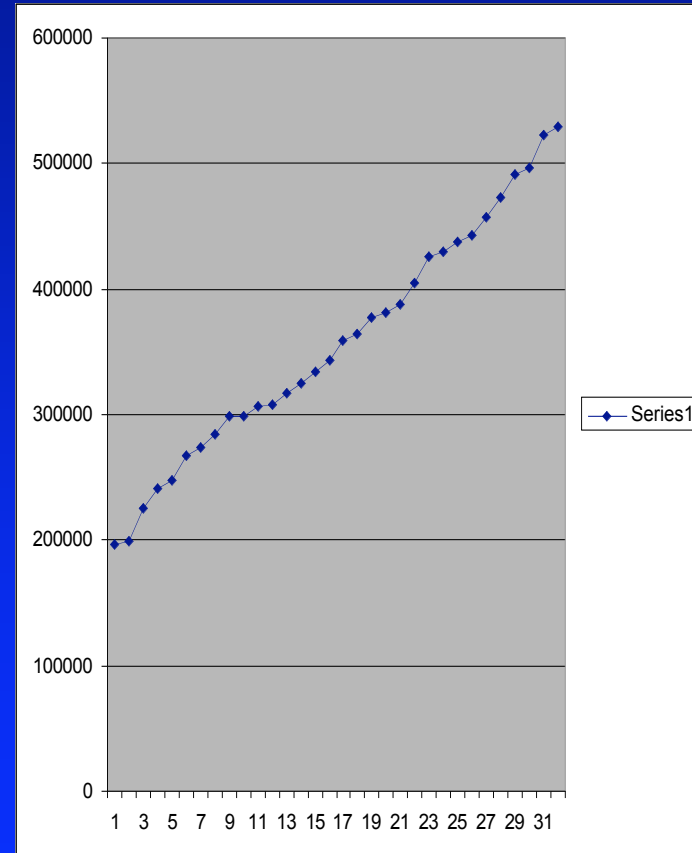
Pilot Study: part 2

- Looked at a single gene (approx 600kb) to compare in-house SNP discovery with the Assays-on-Demand products from AB
- Use 1,500 samples to assess quality of assays and optimisation of reagent use
- Group had previously identified 26 useful SNPs via in-house SNP discovery methods (lengthy process)
- AoD web based search initially identified 23 SNPs in July 2002 (32 in Jan '03)
- Only 1 SNP was an exact overlap

Pilot Study: part 2

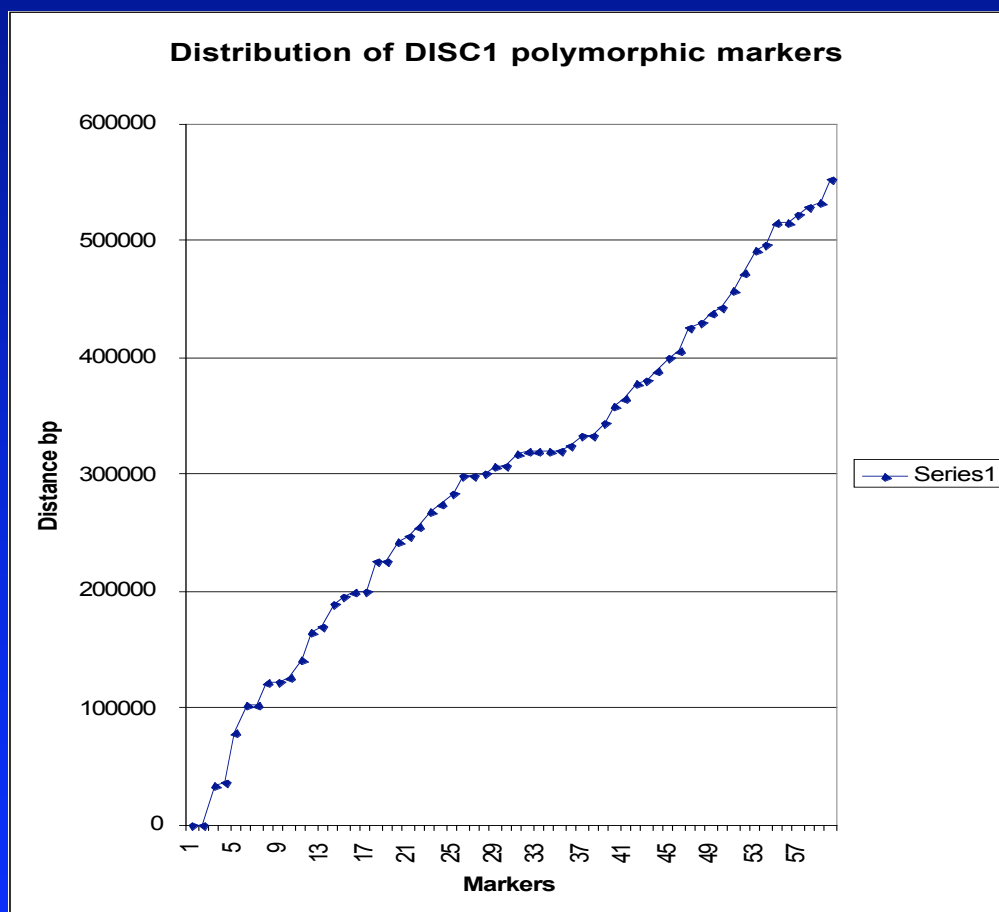


In House



AB TaqMan AoD Markers

Pilot Study: part 2, cont.



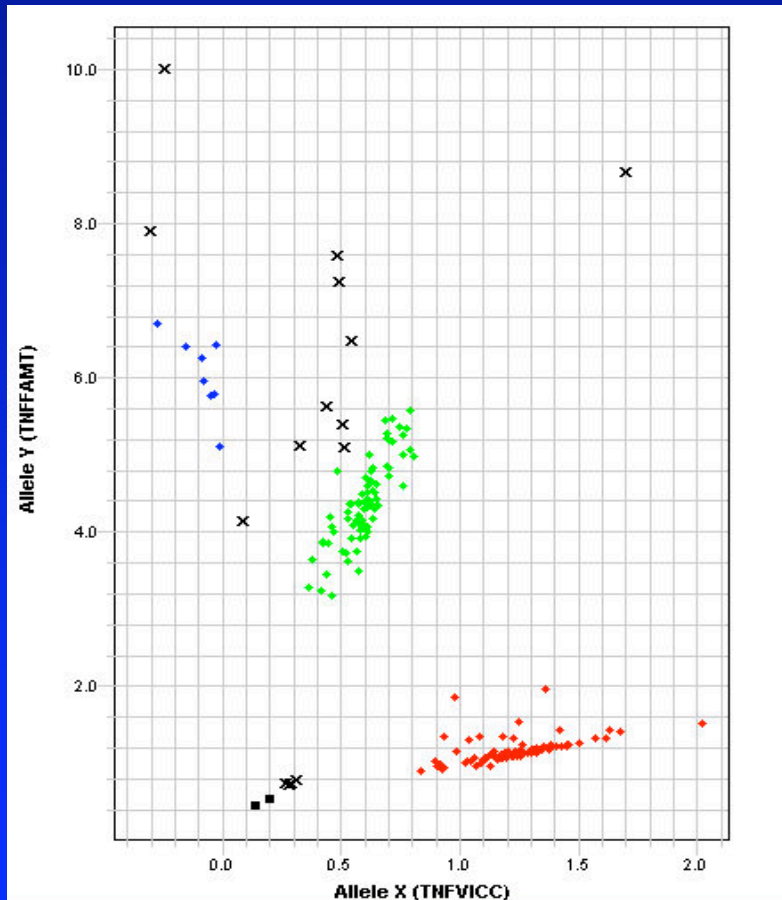
Combined In House and AB AoD

Pilot Study: part 2

Assays-by-Design

- Identified SNPs that are not available as AoD
- Provided sequence data according to AB recommendations
- To date 28 assays submitted
- 24 assays returned
- All failures occurred at the functional test stage (all were designed)
- Used Biomek FX for set-up to 5ul total volume
- Used 'off-line' PCR with MJ Dyads
- Ran 1 plate of all assays initially as RT-PCR to obtain more 'diagnostic' information during run – if all was OK plate is simply read at end-point and data used
- Further problems have been encountered in the laboratory with problematic AbD

Pilot Study: part 2, AbD



- Formatting software helps greatly with on-line ordering
- 1st round failures makes turn around slow
- Easy to use – reagents provided as single tube
- Assay to assay variability observed when typing the same DNA
- Improved time scale for result delivery

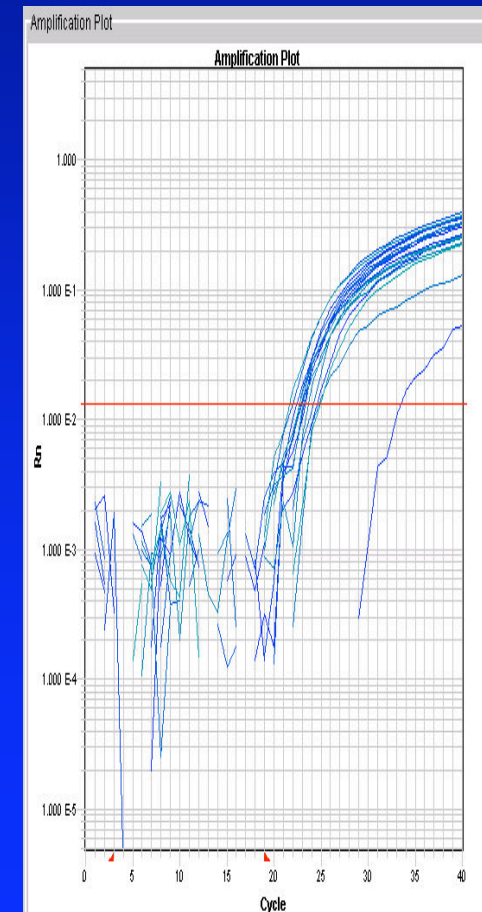
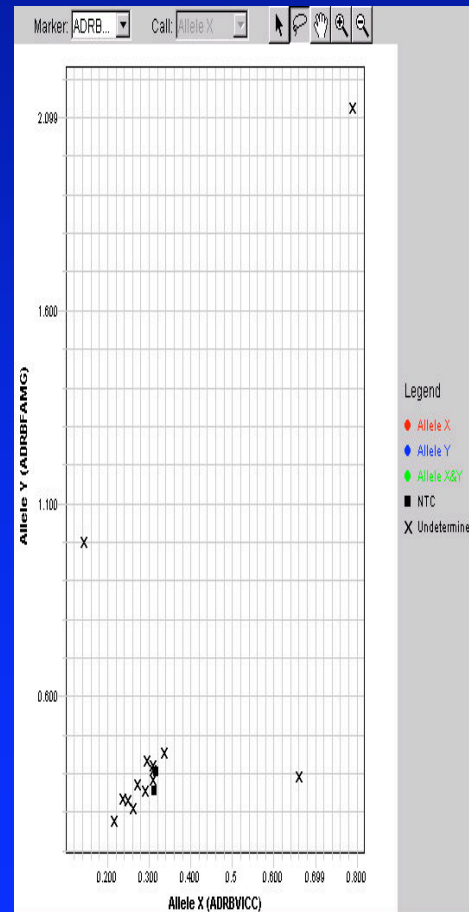
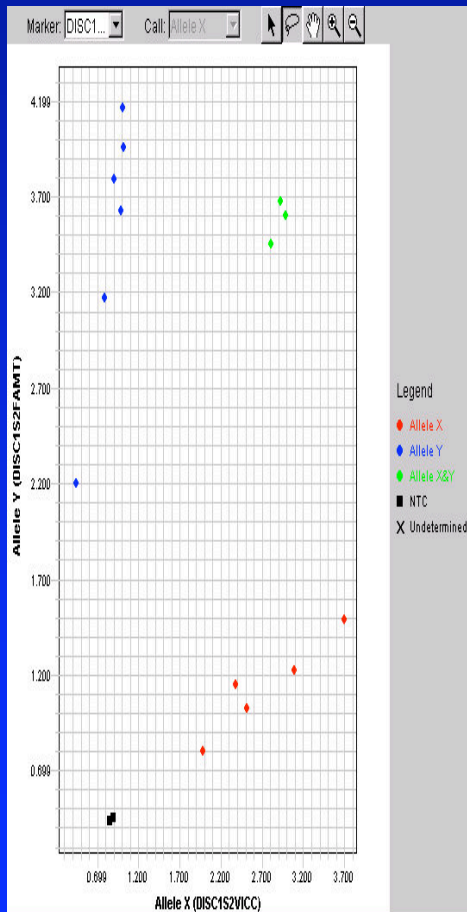
Pilot Study: part 2 summary

- 5ul assay set up works well using Biomek FX (even as RT-PCR runs)
- Off-the-shelf assays work very well producing very good data
- Failure rate of AbD service is higher than expected and not all assays can be used 'out of the box' and we have implemented in-house testing of AbD assays
- Quantitation of genomic DNA is very important to obtain good clusters - currently use picogreen may swap to RNaseP assay
- Quality of DNA equally important as some assays are not tolerant of poor quality DNA
- Interpretation of data is straight forward if clustering is good

Problems with assays

- Problems have mostly been associated with Assays-by-Design
- To date problems associated with 3 AbD from a total of 24 returned (12.5%)
- 1 AoD has given unusual results
- All but 1 assay now working to some degree but problematic assays seem more unstable than those that work first time

Example Assay: ADRB2



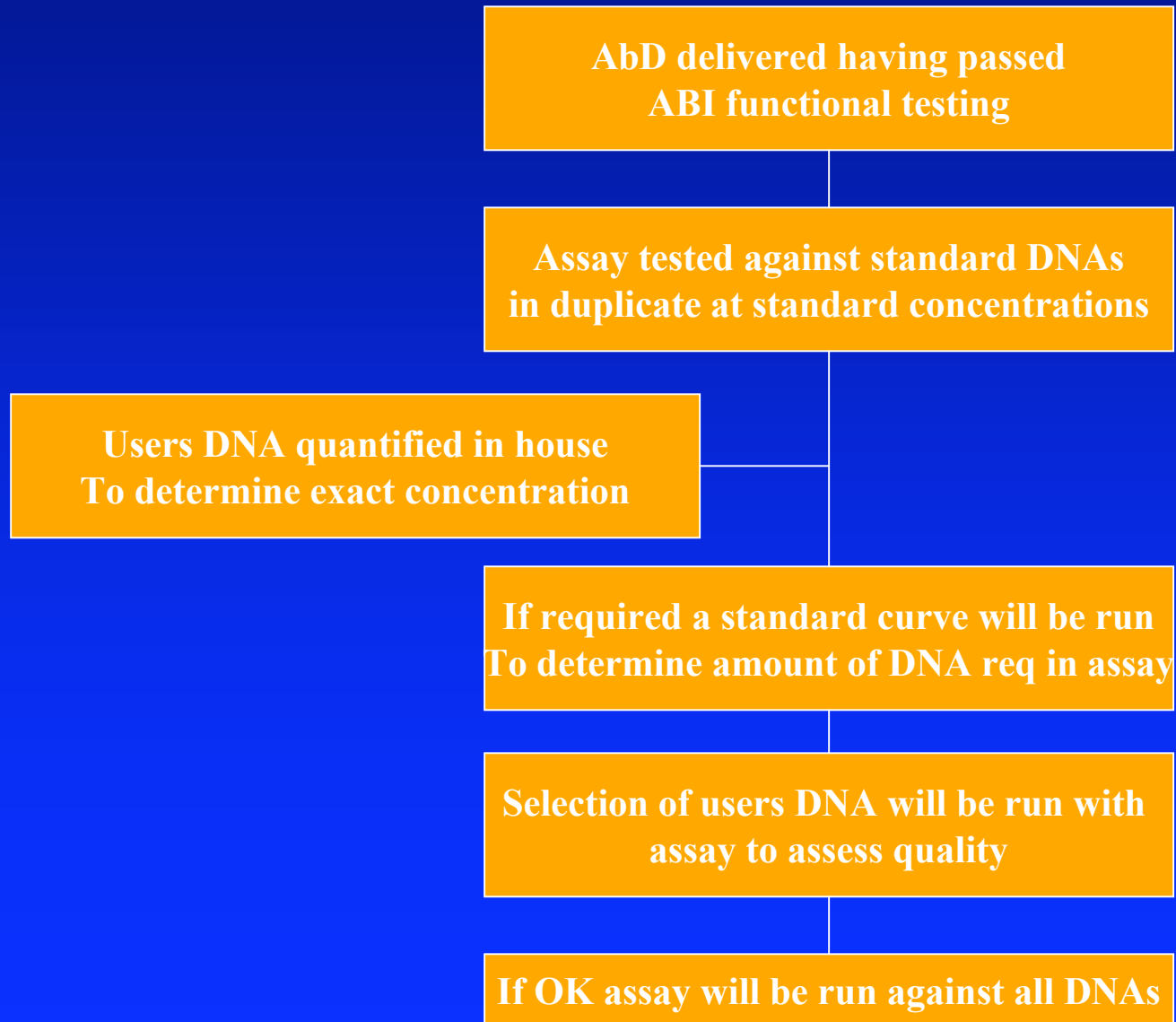
ADRB2 troubleshooting

- Could be a problem with either the DNA or the assay
- Suspected PCR inhibitor in DNA
- Ran real-time PCR on serial dilution of input genomic DNA mass
- Result showed assays with more input DNA worked rather than the more dilute samples as perhaps expected

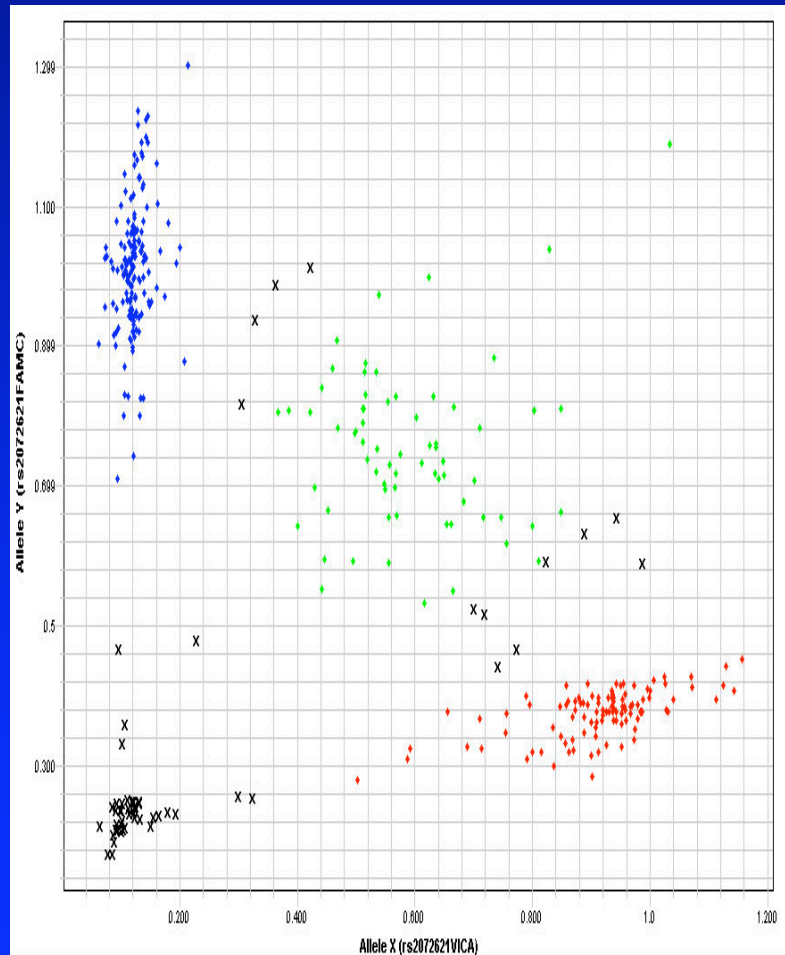
ADRB2 summary

- Given correct input mass with ADRB2 assay results can be achieved (forced reaction, >30ng), however the ADRB2 assay is more inconsistent than others we use
- DNA quality is an issue (A260/A280 1.5-1.7 for failed samples)
- It is the combination of the specific assay and DNA that is important as the same DNA works well with other assays
- The more 'robust' assays are more forgiving of poorer DNA quality
- Currently no information on assay 'robustness' is available when they are designed/manufactured (related to PCR efficiency of the assay?)
- We have yet to establish the boundaries of DNA quality
- May be easier to type these SNP on a different platform rather than spend time trouble shooting & optimising

Processing pathway for AbD assays

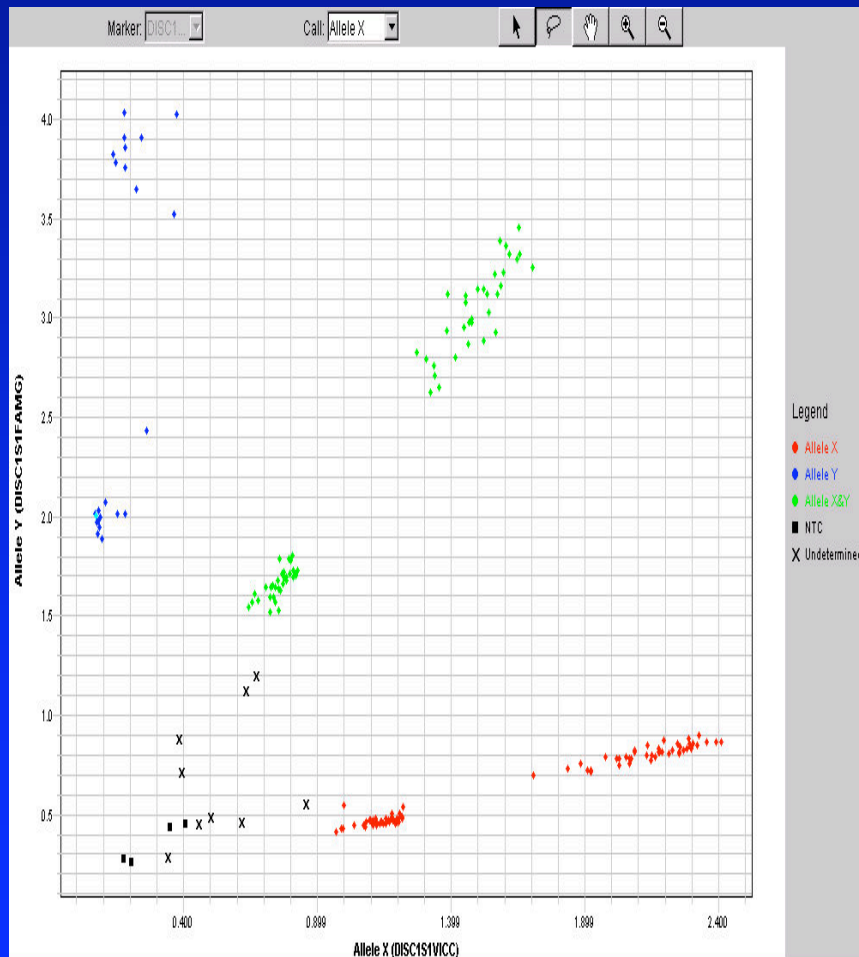


Assays-on-Demand problem?



- Noticed greater spread on clusters at end-point
- DNA clusters well with other assays
- Causes difficulty when calling data
- Only example seen so far
- Assay to be removed from AoD set?

Reagent Optimisation



- Can use X1/2 probe primers
- Can use X1/2 master mix (more dropouts?)
- Only worth running once assays are fully tested in house
- Can spend more time optimising than running all samples against X1 reagents
- Need to assess staff costs vs. reagent costs

In Summary...

- 7900HT provides a fast, accurate, simple to use and cost effective entry level platform for SNP typing
- Works well in central facility as the instrument is easy to use
- Assay set up is easy to automate
- Assays-by-Design and Assays-on-Demand are useful in saving time as most do not require optimisation (project times now measured in weeks rather than months)
- Still require an alternative platform as not all SNP will be available for typing
- Data interpretation is easy
- Ability to multiplex and pool would be useful
- Cost savings can be realised by cutting diluting reagents, however this impacts on project/staff time

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