Optimising Your Genotyping Data: A Practical Guide

At 3CR Bioscience, we are dedicated to your success and committed to supporting you throughout your genotyping journey. Our PACE® Genotyping Master Mix and PACE Genotyping Assays are trusted by thousands of labs worldwide. While our technology is robust, genotyping reactions have multiple variables, and troubleshooting is sometimes necessary. This guide provides insights into the most common issues and solutions to ensure high-quality data production.

Our team of experts is available to assist you at every stage, from assay design and setup to data analysis. From validation to implementation, we help troubleshoot and optimize your genotyping workflow. Troubleshooting is a core part of what we do, and we have encountered and resolved a vast range of genotyping challenges over the years. Our goal is to equip you with the skills and knowledge necessary to generate high-quality data efficiently and consistently.

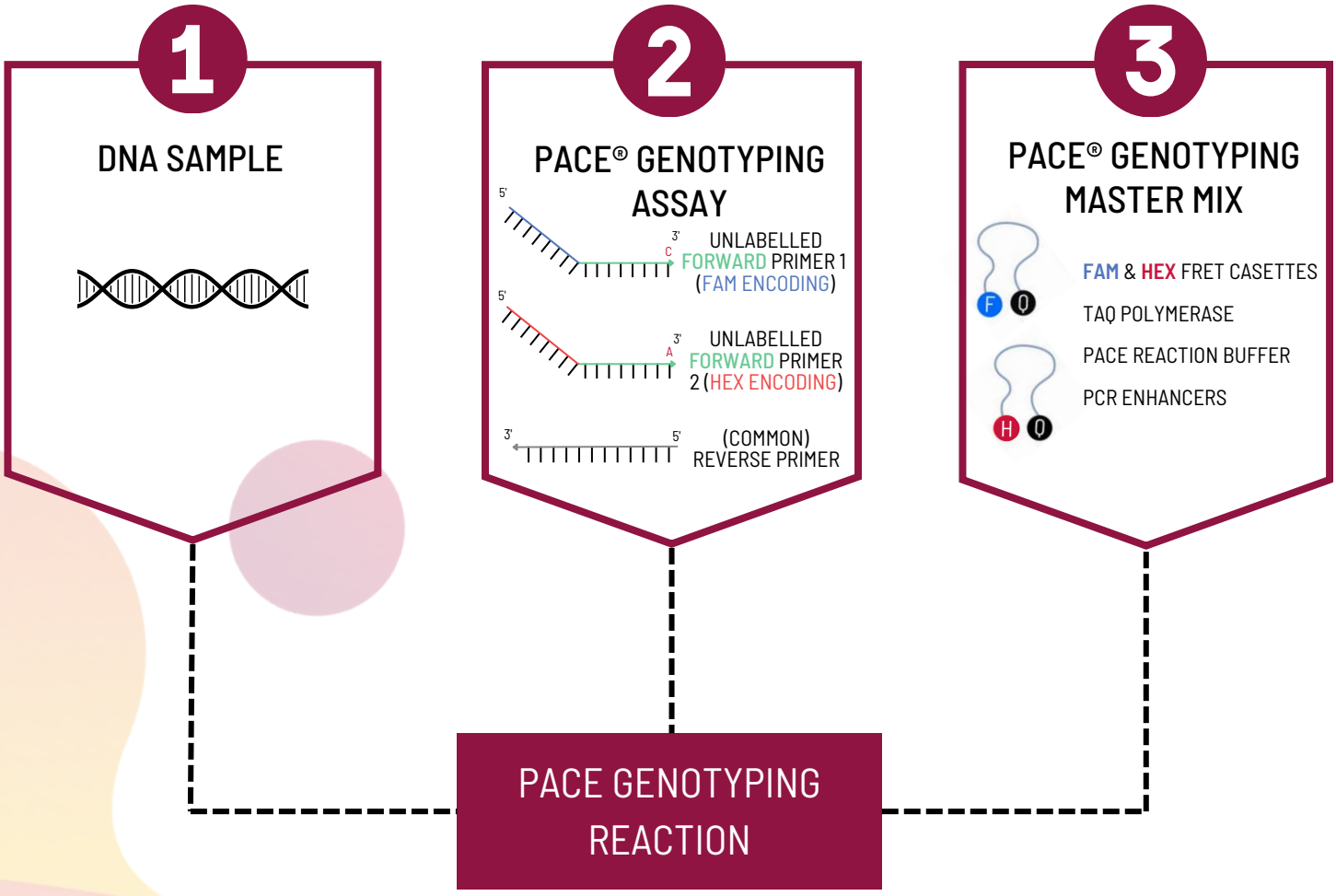


Figure 1: *Components of a PACE Genotyping Reaction*

PACE GENOTYPING TECHNOLOGY OVERVIEW

PACE genotyping technology typically utilises three primers:

- **Two forward primers** (each with a 5′ tail sequence, template sequence, and discriminatory SNP base)
- **One common reverse primer**

The 5′ region of the forward primers contains unlabelled sequences designed to activate the universal signal in the master mix. For more details, refer to our PACE Genotyping Master Mix User Guide and Tech Note: Top 5 Assay Designs for SNP and Indel Genotyping.

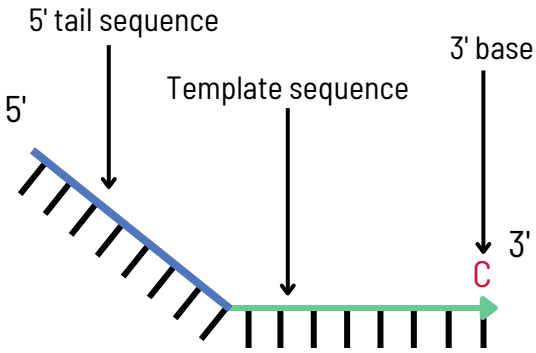


Figure 2: *Allele-specific forward primer in detaill*

Common PACE Genotyping Assay Designs

1. Biallelic SNPs
2. Single-base Indels
3. Small Indels
4. Large Indels with known junctions
5. Large Indels without known junctions

PACE Multiplex Master Mix offers enhanced flexibility with up to four targets per reaction, enabling:

- Two biallelic SNPs in one reaction
- 3- and 4-allele SNP detection
- Three target genes + reference/housekeeping gene

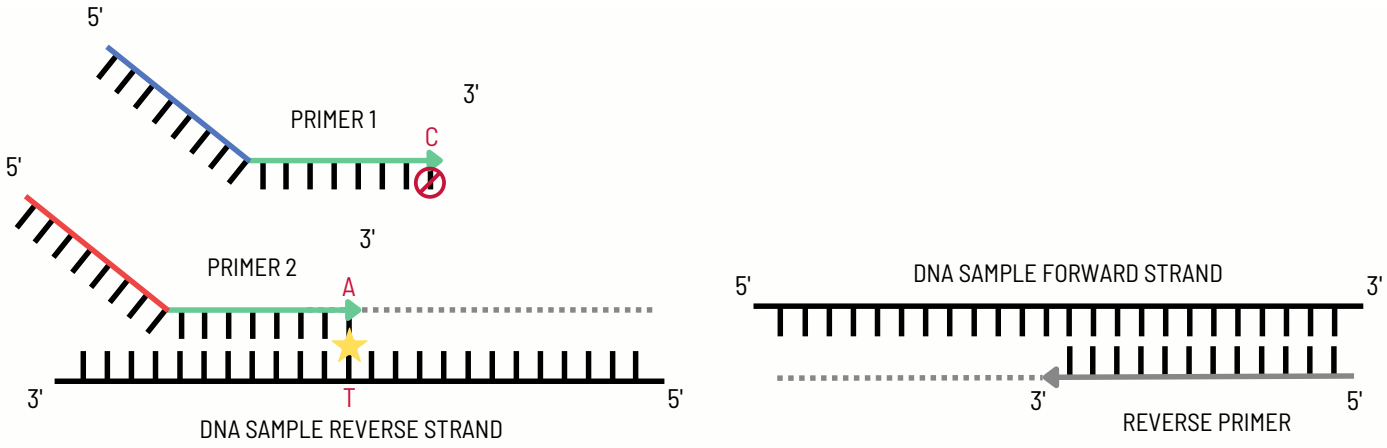
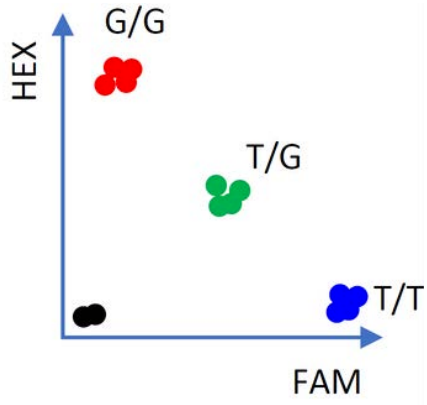


Figure 3: *PACE Allele-Specific Primer Annealing and Incorporation*

INTERPRETING GENOTYPING PLOTS



- FAM-tailed primer detects T allele (homozygous T/T = blue)
- HEX-tailed primer detects G allele (homozygous G/G = red)
- Heterozygous T/G samples appear green (FAM and HEX signals combined)

Figure 4: *A typical bi-allelic SNP detection data plot*

At the point of detection, the quenched, fluorescent reporting cassettes in the PACE Genotyping Master Mix bind to their appropriate reporter-tail sequence complements, and as they unfold become unquenched and emit a fluorescent signal. If the genotype of the target SNP is homozygous, only one of the two fluorescent signals (FAM or HEX) will be generated, and if the SNP is heterozygous, both fluorescent signals will be emitted and a mixed fluorescent signal generated. The signal can be detected either on a qPCR machine or with a fluorescent plate reader (see Figure 5).

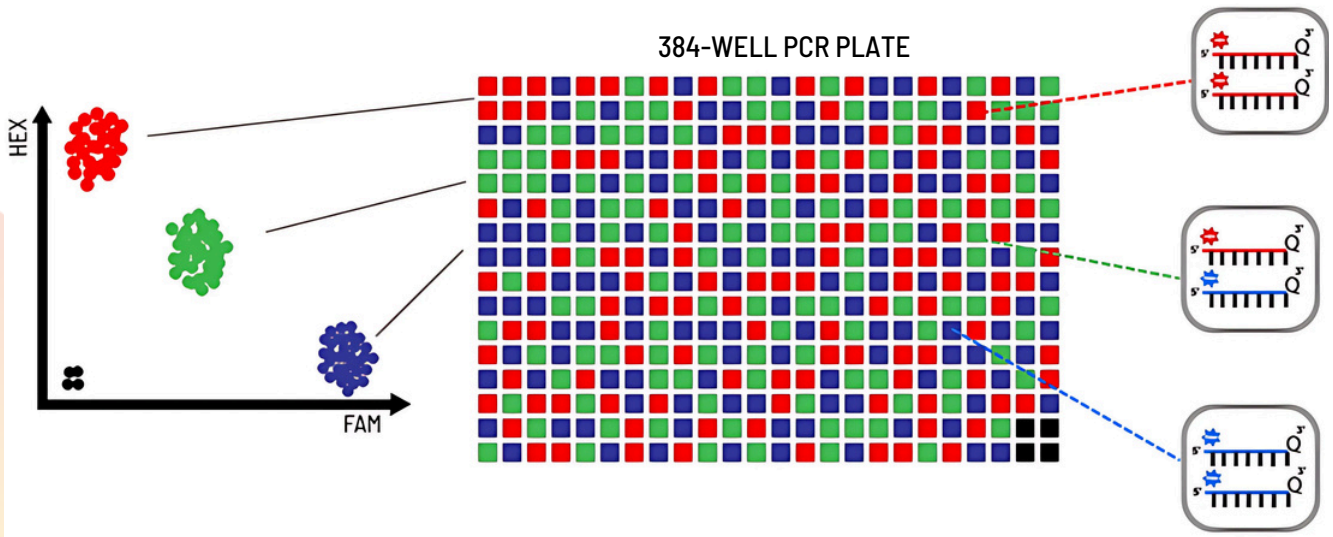
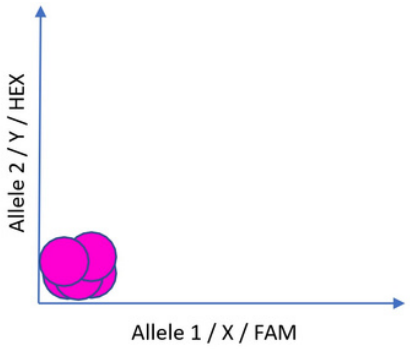


Figure 5: *Cluster plot from a 384-well fluorescent plate read following a PACE SNP genotyping reaction. Red = homozygous HEX sample; Blue = homozygous FAM samples; Green = heterozygous HEX/FAM samples; Black = no-template controls (NTCs)*

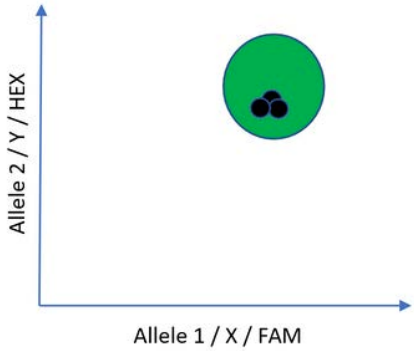
TROUBLESHOOTING COMMON GENOTYPING ISSUES

1. No Amplification



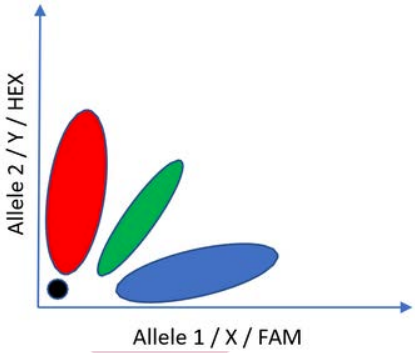
- Insufficient or degraded DNA
- High inhibitor levels - try diluting DNA and/or PACE 2.0 for improved robustness.
- EDTA concentration too high (>0.1mM)
- Incorrect assay mix or missing reagents
- Thermal cycler programming errors

2. All Samples Amplify as Heterozygous



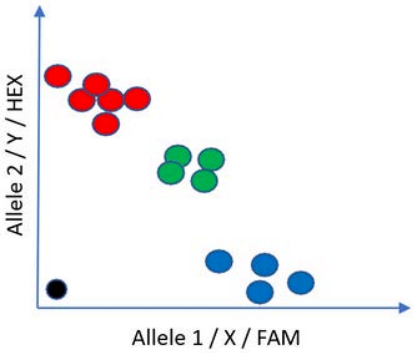
- Improper mix storage
- Primer synthesis issues
- Incorrect primer design or sequence
- Contamination from bleach-based cleaning agents

3. Slow Amplification



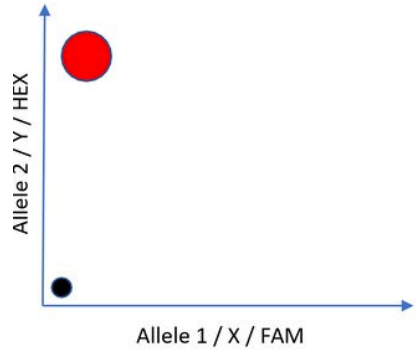
- Insufficient PCR cycles
- Variable DNA quality/quantity
- Excessive EDTA (>0.1mM) – compensate with MgCl2

4. Scattered Clusters



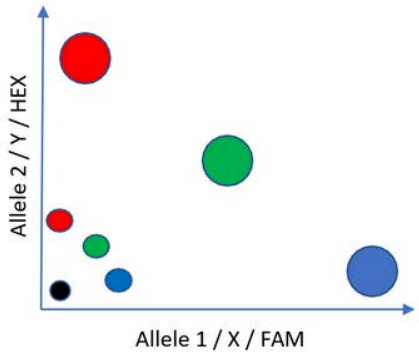
- Cross-contamination
- Insufficient PCR cycles
- Variability in DNA source

5. Monomorphic Clusters (Single Genotype Observed)



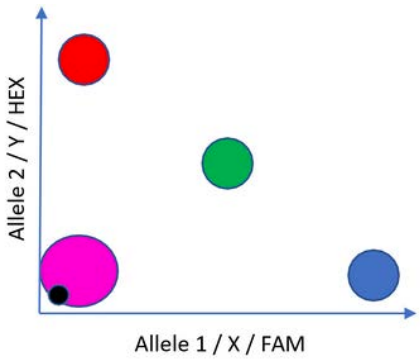
- Samples contain only one genotype for the SNP

6. Distinctly Different Cluster Groups on the Same Plot



- Variation in sample sets or extraction methods
- Differences in ROX concentrations
- Dispensing issue
- Assay efficiency variation - if viewing different assays on the same plate

7. Subset of Samples Not Amplifying Well



- Differences in DNA quality/quantity
- DNA arraying issues
- Master mix dispensing errors
- SNP absent in some samples

CHECKLIST IF A WORKING ASSAY STOPS PERFORMING

If an assay that was previously working well suddenly fails, check the following variables in your process:

1. DNA Quality and Concentration

- Any changes in DNA extraction methods?
- EDTA concentration in buffers?

2. Assay Integrity

- New batch issues (poor oligo synthesis, incorrect design, improper storage, incorrect assay assembly)

3. PACE Master Mix Handling

- Incorrect storage, thawing, or mixing
- Wrong ROX level used

4. Thermal Cycling Program

- Is the correct program being used?
- Issue with thermal cycler

5. Plate Reader Configuration

- Any changes in setup, position, or calibration?
- Post-read temperature should be <40°C
- Any other changes to the process?

CONCLUSION

Successful genotyping requires attention to detail at every step. By following this troubleshooting guide, you can quickly identify and resolve issues in your process, ensuring reliable and reproducible results. For additional support, contact our expert technical support team — we're here to help!