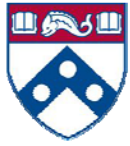


Analytic Approach for Complete and Mixed Engraftment Analysis using Commercial Software



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Background

- ChimerMarker™ (SoftGenetics®) is a commercially available software package designed to integrate genotyping, analysis, and chimerism calculations by short tandem repeat (STR) polymerase chain reaction (PCR)
- The software facilitates chimerism calculations at multiple STR markers (loci), with the goal of providing a more accurate and sensitive result than single locus analysis
- We present the evaluation, validation, and implementation of ChimerMarker™ into the workflow of a clinical laboratory for post-transplant chimerism analysis by STR PCR

Goals of the Study

- Compare the use of ChimerMarker™ software calculations at multiple markers to a manual, single locus calculation method
- Develop protocols for chimerism analysis using ChimerMarker™ software including the creation of a pre-transplant project (pre-project) and post-transplant analysis

Methods

- 13 cases (0-100% recipient) used for software validation with comparison to a manual, single locus calculation method
- PCR with Powerplex 16 HS System (Promega), followed by capillary electrophoresis (ABI 3500)
- Pre-projects including a duplicate donor (D) sample (D1Baseline) and second recipient (R) sample (RBaseline, usually buccal cells) were created for each R/D pair by applying novel algorithms developed to include or exclude informative markers based on their potential to be affected by stutter or other artifacts that would decrease analytic sensitivity or specificity for donor and recipient (Figure 1)
- Data from 12 R/D pairs were gathered to calculate the average percent stutter at each marker and allele in the cohort (n-1, n-2, and n+1 positions), which were then used to set marker-specific stutter filtering and adjustment
- Post-transplant samples (blood, T cells, or bone marrow) analyzed using the selected markers from the pre-projects
- DNA input was varied for 9 patients
- Chimerism results for 120 patients were compared between ChimerMarker™ using selected markers and the manual, single locus method
- Cutoffs determined for lower limit of detection

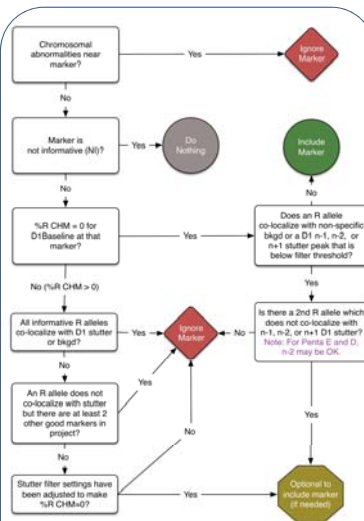


Figure 1. Donor Baseline Locus Selection Algorithm. This flowchart was used at each locus to determine if the locus would be included or ignored in the software calculation. A similar algorithm was developed for Recipient Baseline (not shown).

References and Acknowledgements

- Chen, D.P., C.P. Tseng, S.H. Tsai, T.L. Wu, P.Y. Chang, and C.F. Sun. Systematic analysis of stutters to enhance the accuracy of chimerism testing. *Ann Clin Lab Sci*, 2008. 38(3): p. 264-72.
- Kristi, D. and T. Klein. Reliability of quantitative chimerism results: assessment of sample performance using novel parameters. *Leukemia*, 2006. 20(6): p. 1169-72.
- ChimerMarker™ Manual

Results

Stutter Varied From 1-10% At Each Locus

- Marker-specific stutter filtering was set based on results of stutter analysis in 12 patients, with average stutter % ranging from 1-10% (Table 1)

Marker	Chr Loc	Repeat Motif	Bin Range (Repeats)	STUTTER % BY POSITION		
				n-2	n-1	n+1
D3S1358	3p21.31	[TCTA][TCTG]	12-19	1	10	2
TH01	11p15.5	AATG	4-13.3	1	4	1
D21S11	21q21.1	[TCTA][TCTG]	24-38	1	10	3
D18S51	18q21.33	AGAA	7-27	1	8	2
Penta_E	15q26.2	AAAGA	5-26	1	4	1
D5S818	5q23.2	AGAT	7-16	1	8	2
D13S317	13q31.1	TATC	8-15	2	8	3
D7S820	7q21.11	GATA	6-15	2	6	2
D16S539	16q24.1	GATA	5-15	2	8	2
CSF1PO	5q33.1	AGAT	6-15	1	7	2
Penta_D	21q22.3	AAAGA	2.2-17.1	1	2	1
vWA	12p13.31	[TCTA][TCTG]	11-24	2	9	2
D8S1179	8q24.13	TCTA	8-19	1	8	2
TPOX	2p25.3	AATG	6-13	1	4	1
FGA	4q28	complex	17-51.2	2	10	2

Table 1. Average % stutter for each locus. Since the software only corrects for n-1 stutter, manually excluding loci with n-1, n-2, and n+1 stutter markedly improved result accuracy.

Pre-Project Creation with Manual Locus Selection

- A pre-project for each R/D pair was created using D1Baseline sample and the donor algorithm shown in Figure 1 to select markers (Figure 2)
- In our unique approach, analysis of results is dependent upon analyzing donor and recipient (blood and buccal swab) samples in duplicate; one as known R and D samples and the second simulating post-transplant samples for R and D

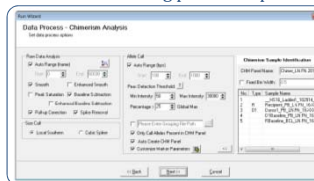


Figure 2. Screenshot of ChimerMarker™ Run Wizard Settings applied to single-donor chimerism projects.

- Loci with n-1, n-2, and n+1 were manually excluded, resulting in improved result accuracy for calling a donor sample correctly as 100% donor (Figure 3)
- A similar approach was used with RBaseline to select markers that correctly identified a recipient sample as 100% recipient
- Deconvolution (calculations using shared homozygous alleles) was not used except when necessary because it is less adaptable across a wide range of % recipient (Figure 4)

Benefit of Algorithmic Locus Selection

Figure 3. Comparison of pre-project donor baseline without (left) and with (right) marker selection using algorithm from Figure 1. Compare red boxed results. Loci with R alleles in donor stutter positions are excluded (right), giving true 0% recipient result in a known 100% donor sample (D1Baseline).



Sensitivity Down to 1% & Strong Correlation with Manual Method

- DNA inputs of 2 ng were more reliable, with lower %CV, less bias, and less allelic imbalance or allele dropout than 1 ng (data not shown)
- Analytic sensitivity of software reliably detected donor or recipient DNA to at least 1%
- Peak score cut-off of 0.5 maximized analytic sensitivity (96.0%) and specificity (92.5%); manual inspection of scores of 0.5-7.0 still needed to rule out artefactual background
- On average, four markers were used (range 2-12) per sample for calculation in ChimerMarker™ and correlation with the manual single locus calculation method was strong (Figure 5)
- Algorithms also applied to double donor transplants (not shown) but needed adaptation to account for all possible engraftment states (e.g. D1 only, D2 only, D1+D2)

Figure 4. Examples of deconvolution locus selection for donor baseline samples (ability to detect small amounts of recipient).

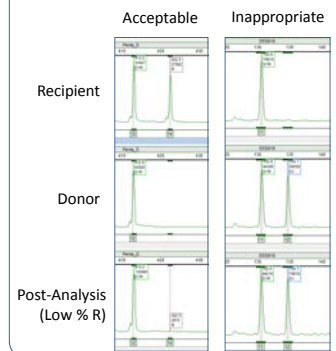
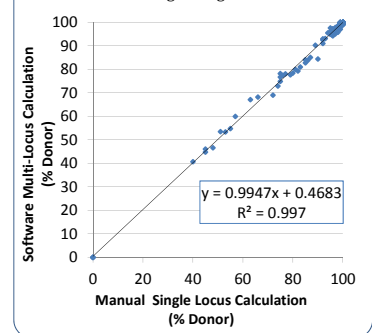


Figure 5. Comparison of 120 patients measured by the manual, single locus method and ChimerMarker™ multi-locus method showing strong correlation.



Conclusions

- Analysis of multiple loci using ChimerMarker™ is optimized through the use of an algorithm for locus selection developed by our laboratory
- Pre-selection of markers for analysis minimizes the effect of stutter and other artifacts providing a reliable, sensitive, and reproducible approach to post-transplant chimerism analysis
- Modification and validation of ChimerMarker™ software is necessary for successful integration within a clinical laboratory workflow
- Overall, these alterations empower the software to detect complete engraftment equal to or better than a manual, single locus method