Oligo Design Across the Mouse Genome

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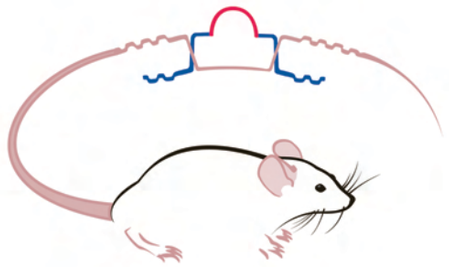
Abstract: Fluorescence-quenched probes are routinely used to gauge gene copy number. We describe a bioinformatic engine for the design of such oligos, and used to generate five thousand qPCR assays for the NIH Knockout Mouse Project (KOMP). Here, we demonstrate the performance of a subset when amplified from wild-type mouse DNA. Analysis of this data-set uncovers important trends in amplification performance and emphasizes the need to screen assay specificity using both bioinformatic and empirical a-proaches. Redundancy and accessibility are considerations that become pronounced in large-volume sequence design. Based on this experience as well as user feedback, new software functionality is introduced to improve upon these qualities.



Loss of Native Allele Assay

The Velocigene® division of Regeneron employs Bacterial Artificial Chromosomes to recombine across the genes of interest. Detailed methods on creating these complete null mutations can be found:

Valenzuela, D. M., et al. (2003). High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. Nat Biotechnol 21, 652-659.

Knockout is confirmed by qPCR, comparing the amplification of six reference genes against two assays that target the beginning and end of each gene. To date, the total assays generated by RealTimeDesign and tested by Regeneron are as follows:

> 5000 assays synthesized.
• 93% success for new designs.

> 4500 tested for performance.
•†80% success for re-designs.

Design rules are incorporated in three parameter sets of decreasing stringency. RealTimeDesign automatically applies them in series, all though challenging targets may require deviating from the default.

- 87% of all assays designed using three default parameter sets.
- The Most Restrictive parameter set yields a 96.7% success rate.
- The Less Restrictive parameter set yields a 96.4% success rate.
- The Least Restrictive parameter set yields a 94.4% success rate.
- Assays that cannot be designed under the above parameters yield an 83.6 % sucess rate.

Assay sequences that have been tested with success are available to view at
www.KOMP.org & www.velocigene.com/KOMP/search

Methods

KOMP Assays are initially designed using the Express Mode of RealTimeDesign operating under default parameter sets. Assay specificity is then confirmed using the "electronic PCR" tool available through the National Center for Biotechnoll ogy Information. Genes that fail to yield an assay under Express Mode, or those that reveal off-target hits by ePCR, are then designed using the Custom Mode of RealTimeDesign with parameter values that deviate from the default. For the purpose of this poster, the performance of assay pairs are screened by amplifying **mouse** genomic DNA (Promega cat. no. G3091) in triplicate at a quantity of 3.75 ng / reaction. PCR is performed on the Rotor-Gene 6000 using the following thermal cycling conditions: 95 °C for ten min. followed by 40 cycles of 95 °C or 20 s, 60 °C for 60 s.

Reaction Components	Volume	Final Conc.
• Sample Template	3.00 µL	N/A
• 2X Immomix (Bioline)	10.0 µL	1X
• Each Forward Primer (10 µM)	5x 0.60 µL	300 nM
• Each Reverse Primer (10 µM)	5x 0.60 µL	300 nM
• Each Probe (10 µM)	5x 0.20 µL	300 nM
Total Volume	20.0 µL	

Assay Name:	Oligo Sequences:
Gsdma2-10517TU	Forward: TTCCTTACCACAGATCCAACAC
Custom Parameters	Reverse: CACAGTCCCCTTTACCTTCATCA
Probe: [FAM]-ATGCTGGATGTCAGAGTAGAGGGAGATGT-[BHQ1]	

Assay Name:	Oligo Sequences:
Gsdma2-10517TD	Forward: AGCCTTCCCGGCCTTACTG
Most Restrictive	Reverse: ACTGCCTCTGCCTCCTGACTG
Probe: [FAM]-TGCTCTGTCTGAAACACTCCAAGATGGT-[BHQ1]	

Assay Name:	Oligo Sequences:
Bmp8a-10245TU	Forward: GAAGACCAAGGGCGTGAAG
Less Restrictive	Reverse: AAGATGCCCAGGCCGAAGTC
Probe: [FAM]-CGCTGCCCCACCGCCTTATCC-[BHQ1]	

Assay Name:	Oligo Sequences:
Bmp8A-10245TU-2	Forward: TATCTGCTCTCCGCCCACC
Custom Paramters	Reverse: ACGGTAGACCCGTCCTTGAC
Probe: [FAM]-CGCTGCCCCACCGCCTTATCC-[BHQ1]	

We would like to acknowledge Dean Fiala, Raymond Peterson, and the rest of Celadon Laboratories, for developing the code that powers RealTimeDesign. We also thank Yingzi Xue, Rostislav Chernomorsky, David Frendeway and others in the Velocigene® division of Regeneron.

Example Assays

Redundancy is key since the read-out from qPCR assays occasionally mislead. Although designed under different paa rameter sets, the agreement between two assays targeting the gene Gsdma2 (right) typify the majority of assay pairs designed for KOMP.

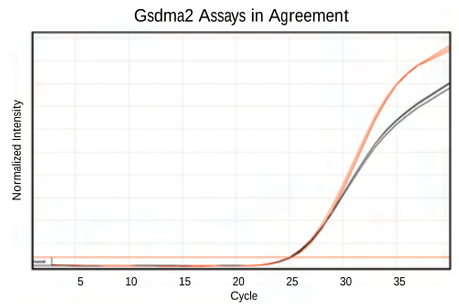


Figure 1: assay 10517TU (red traces) and 10517TD (black traces) both target the gene Gsdma2 and demonstrate equivalent C_T values when amplifying from 3.75 ng of DNA.

Newly-developed qPCR assays are screened against a known quantity of wild-type DNA and fail if they do not amplify within two cycles of expected. Assays that co-amplify gene homologs might cross the threshold too early.

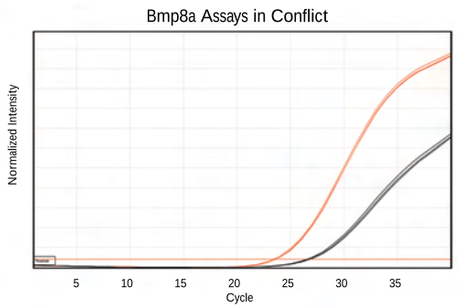


Figure 2: assay 10245TU (red traces) co-amplii fies multiple Bmp homologs and crosses the cycle threshold earlier than expected. The ree design of this failed assay (black traces) specifii cally targets Bmp8a to the exclusion of others

Conclusion

RealTimeDesign plays a critical role in the KOMP by rapidly generating oligo sequences to mouse gene targets The testing of this collection of assays indicates a good rate of success and offers important insights into the PCR process: specificity must be screened in advance or else become the principal reason for amplification failure. Common design guidelines such as ball anced GC content, short amplicon length, and minimal misalignments are built into the software's algorithms. Their importance is underscored by the increasing rate of success corr related with the increasing stringency of parameters. But in a testament to the robust PCR mechanism, any one of these guidelines can be discarded to generate a functional assay still, as confirmed by those assays designed on a custom basis. RealTimeDesign is available through the web, it processes on a remote server to avoid consuming local computer resources, and arr chives each design into a unique database for every user. These qualities have proven essential for high-throughput projects such as the KOMP

REALTIMEDESIGN™

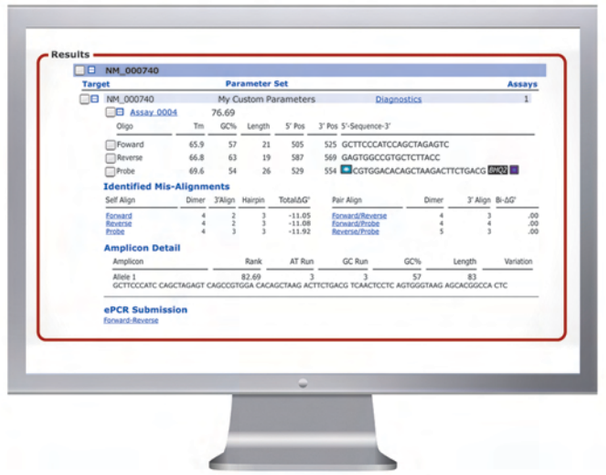


Figure 3: screenshot from RealTimeDesign™, showing oligo sequences and their properties. This software is available for free public use on the web at:

www.qPCRdesign.com

RealTimeDesign is a web application for oligo sequence design and used to generate primers and probes for the KOMP in a rapid and automated fashion. The following software features play a critical role in this and other high-throughput projects:

- Numerous genes are processed simultaneously in batches.
- A user database stores the history from 100 different designs.
- Parameters can be customized and made default for the future.

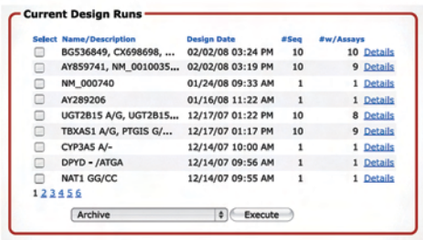


Figure 4: screenn shot from RealTii meDesign™, showw ing the design hiss tory of multiple batch runs. Imporr tant records can be archived and exx ported for future reference

New features developed specifically for the KOMP:

- †Alternate assays are now quickly designed at the click of a button when the original is found to fail performance testing.
- †BHQ*plus*™ probes are now proposed for challenging targets that cannot accommodate traditional dual-labeled probes.

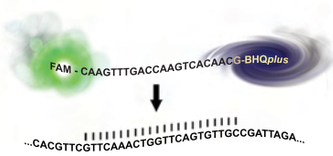


Figure 5: BHQ*plus* probes contain a duplex stabilizing chemistry to allow shorter oligo sequences that bind at the proper temperature