Rutgers, NJMS Team Describes Multiplexed 'SuperSelective' PCR Primers for Rare Mutation Detection

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NEW YORK (GenomeWeb) – Researchers at Rutgers University and the New Jersey Medical School have published a description of "SuperSelective" PCR primers that enable detection of extremely rare SNPs in a background of wild-type DNA and demonstrated how they can be used for quantitative multiplex cancer-related assays.

The primers — which can detect as few as 10 mutant copies of a gene in a background of 1 million wild types in a singleplex assay — were initially described at the 2013 Cambridge Healthtech Institute Molecular Medicine Tri-Conference, as reported by GenomeWeb.

But the current iteration, published last week in *PLoS One*, has many advances, Fred Kramer, a researcher in the department of microbiology, biochemistry, and molecular genetics, and principal investigator on the study, said in an interview.

Namely, the method uses tag sequences and molecular beacon probes to parse out the different amplicons generated in multiplex assays. It also has enhanced specificity and signal-to-background, uses a reference gene, and incorporates a way to separately quantify how much of each target was present in the original sample.

The method is as simple as standard PCR, and can be deployed on any standard spectrofluorometric thermal cycler, Kramer said.

The Kramer lab may be most well-known as the 1992 inventors of molecular beacon technology. The Rutgers Public Health Research Institute has licensed that tech non-exclusively to more than 75 licensees over the years, and a portion of the income fully funded the current research, Kramer said.

However, potential industry collaborators told Kramer that the original monoplex version of SuperSelective primers would be more useful commercially if it could be used for multiplexing.

"Everyone who we spoke to, especially companies that make diagnostic assays — we talked to, off the top of my head, Abbott, Gen-Probe, BioMérieux, Cepheid, and Qiagen — they all said it is only practical to have a PCR assay if you can do it in multiplex and assess quite a few relevant mutations," Kramer said.

If the many mutations existed on separate genes, or if both mutant and wild type could be amplified and later distinguished with complementary probes, there'd not be a problem, Kramer said. "The real problem is that most of the significant mutations relating to cancer occur in the same gene."

Thus, a few additional years of work has now resulted in primers that can be used for three targets, as published in *PLoS One* last week, but with the potential for many more. Kramer's group has also gone far in exploring the minutiae of the design of the primers, experimenting with varying every parameter to see how that affected the PCR.

One innovation enabling the multiplexing is a tag at the 5' end of each SuperSelective primer. "If an amplicon is generated from that, [the tag sequence] will be incorporated into the 5' end of the amplicon," Kramer explained. Then, when the common reverse strand copies it, the tag element will also be incorporated in the form of a complement.

"We use the complements of these unique 5' tags as the targets for probes," Kramer said. "Since we're totally free to use any unique sequence we want, if you have five or six SuperSelective primers in a multiplex assay, each one can have a totally different 5' tag, which means it will have a totally different complement at the 3' end of the minus strand." These are then picked out with fluorescently labeled probes, specifically the lab's own molecular beacons.

A standard qPCR thermal cycler can then take the spectrum at the end of each annealing stage, and the contribution of each colored probe can be quantified to yield a readout of the amount of amplicon.

Notably, the method also uses a limiting amount of the SuperSelective primers but an excess of reverse primer, which reduces competition for the probes. This also reduced the likelihood of heteroduplex formation, Kramer said, between, say, plus amplicons of more abundant mutants and minus amplicons of less abundant mutants.

The primers have a unique sequence, called a bridge, as well as a sequence called a foot, and these also get incorporated into the complementary strand. Thus, even if there is only one nucleotide different in the foot region, all the amplicons from the other mutants will possess a different bridge sequence complement, so, “The primers breed true, and the unique bridge sequence incorporated into each amplicon assures the specificity of continued exponential amplification.”

Because of the in-depth investigation of primer design variation, the group is also able to tweak them to adjust reaction rates so cycle thresholds can be more directly compared between targets in a multiplex reaction. Comparison to the reference gene can then give the relative abundance of each mutant.

Using the common analogy of a needle in a haystack, some other rare mutant detection methods focus on the overabundant wild-type elements, essentially eliminating the "hay" in some way. But Kramer's method instead might be more akin to using a powerful magnet to grab out the needle. "We can routinely detect five molecules in the presence of 10,000 wild-types [in multiplex assays], which is what we expect … in a liquid biopsy," Kramer said, adding that the actual limit of sensitivity has not been determined but may turn out to be only one or two molecules of a mutant fragment.

The group is now experimenting more, looking at pre-amplification to drive the limit of detection even further.

Detecting and quantifying somatic mutations is, of course, most commonly associated with oncology-

related assays. To validate the method, Kramer and his team developed assays for *BRAF* and *EGFR*. Through this process they were able to demonstrate that components of the chemistry required modifications to best function in different nucleotide contexts.

However, Kramer noted that while the method was hammered out in plasmid-based prototypes that mimicked small bits of circulating tumor DNA in liquid biopsies, "in fact, it could be used for anything," including cerebral spinal fluid, urine, or saliva. It also could be used for rare infectious pathogens, drug-resistant bacteria, or for non-invasive prenatal testing in which fetal DNA is present in a superabundance of maternal nucleic acid.

"We did general explorations of multiplex, selective, and sensitive PCR — now it is time for these techniques to be tried out with clinical samples by people who know the clinical situation," Kramer said.

He has so far contacted about 15 different companies that might be interested in SuperSelective primers for particular mutants.

"We'll help them develop the multiplex assays and they'll test them on real clinical samples," he said, adding that now, with a highly sensitive test, perhaps determining the level of residual disease could be used to adjust treatment protocols of toxic drugs, for example. Kramer also speculated that such a test could be used to monitor or regularly screen people who might be predisposed to cancer to see if they begin to express ctDNA.

In terms of liquid biopsy, a test from Roche that detects exon 19 deletion or exon 21 (L858R) substitution mutations in *EGFR* was approved last week by the US Food and Drug Administration as a companion diagnostic to Tarceva (erlotinib), a drug for non-small cell lung cancers. Kramer noted that SuperSelective primers could potentially be used in an assay like this to increase the number of targets measured in a single test.

Similar approaches to SuperSelective primers have also evolved in other laboratories, Kramer said. In particular, he mentioned Alison Todd's lab in Australia, which is associated with the firm SpeeDx, as well as DPO primers from Korean firm Seegene. These each have some coincidental similarities to Kramer's method, demonstrating that it has commercial appeal.

Kramer's group, meanwhile, has filed a few patents on the method, but he is not concerned with "who invented what where," he said. "All that matters is if we have something that could help patients. It would really be wonderful if a $50 assay could be done monthly on someone [predisposed to cancer] — it would make such a huge difference."