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Application of Quantitative Real-Time PCR for Monitoring the Process of Enrichment of Clones on *In Vitro* Protein Selection

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***In vitro* selection of proteins from cDNA libraries using display technologies, such as the *in vitro* virus method, is a powerful means for the discovery of novel protein interactions. After iterative screening, selected proteins are usually identified and evaluated by cloning and sequencing analysis. Previously we applied real-time PCR for evaluation of the sequences obtained on *in vitro* virus screening. Here, we have presented additional data regarding monitoring of the process of enrichment of selected clones in each round of selection and elimination of false positives by real-time PCR, and have also discussed the utility of the novel method. This approach should also be applicable to other display technologies.**

Key Words: display technology, false positive, *in vitro* virus, mRNA display, real-time PCR.

Abbreviations: IVV, *in vitro* virus; Nrbf2, nuclear receptor binding factor 2; Aes, amino-terminal enhancer of split; Gas5, growth arrest–specific 5.

Display technologies (*1–3*) that link genotype (DNA or RNA) and phenotype (protein) molecules are powerful tools for the discovery of interaction partner proteins for various targets (*e.g.*, proteins, nucleic acids, small-molecular compounds, and drug candidates), using cDNA libraries or artificial random-sequence libraries. Phage display (*4–8*) is the most widely used display technology, and has uncovered many novel functional proteins, protein–protein interactions, and DNA–protein interactions. Furthermore, totally *in vitro* display technologies involving cell-free translation systems, such as ribosome display (*9–11*), mRNA display (*12*, *13*), and DNA display (*14–16*), have also been applied for the discovery of novel functional proteins, screening of drug targets (*17*), and protein–protein interaction analysis (*18*).

Figure 1 is a schematic representation of a typical screening procedure involving totally *in vitro* display technologies. Initial libraries of genotype-phenotype linking molecules are affinity-screened and amplified iteratively. The resulting DNA library is cloned into some kind of cloning vector and then sequences are determined by commonly used methods. However, we cannot easily determine much about the abundance ratio or the process of enrichment of the selected clones in each round from only the cloned numbers of the sequences, although such information would be quite useful for evaluating the clones and for optimizing the selection conditions. In addition, the resulting clones often include false positives that are merely abundant in the initial library or that are accidentally picked up in spite of having no binding activity. Therefore, we planned to apply a quantitative real-time PCR technique to monitoring of the process of enrichment of clones on *in vitro* protein selection and elimination of false positives easily by accurately determining the numbers of molecules of each selected DNA in the resulting libraries to confirm the specific enrichment of the sequences.

We recently performed affinity screening of Jun-associated proteins from a mouse brain cDNA library (*19*) using our *in vitro* virus (IVV) method (*12*, *13*), one of the mRNA display technologies. In this study, we further analyzed 451 clones including 217 previously analyzed clones which had been picked up from the library on five rounds of iterative screening (*19*). Of the 451 clones, 271 (about 60% of the total analyzed clones) were confirmed to represent intact RNA-protein conjugated molecules without any frame-shift or stop codon on sequence analysis. These 271 clones were clustered into 22 independent sequence groups that were considered to be credible candidates for Jun-associated proteins (Table 1); they included three unreported candidates, nuclear receptor binding factor 2 (Nrbf2) (*20*), amino-terminal enhancer of split (Aes) (*21*), and growth arrest–specific 5 (Gas5) (*22*), together with 19 that we had previously reported (*19*).

We performed real-time PCR analysis to determine the numbers of DNA molecules of the 22 selected sequences (Table 1) in the initial cDNA library, and the libraries obtained on each round of IVV screening in the presence [bait (+)] and absence [bait (–)] of the Jun bait protein (Fig. 2). We found that all of the selected sequences except for those of Aes and Gas5 were enriched in each round of bait (+) selection (Fig. 2A), whereas β-actin (negative control) in the bait (+) selection (Fig. 2A), and all sequences in the bait (–) selection (Fig. 2B) were found not to be enriched. These results indicate that 20 of the 22 sequences had been selected specifically on the basis of affinity for the Jun bait protein, whereas Aes and Gas5 are false positives. Thus, of the three new candidates discovered here, only Nrbf2 was concluded to be a true positive. The interaction between Jun and Nrbf2 was

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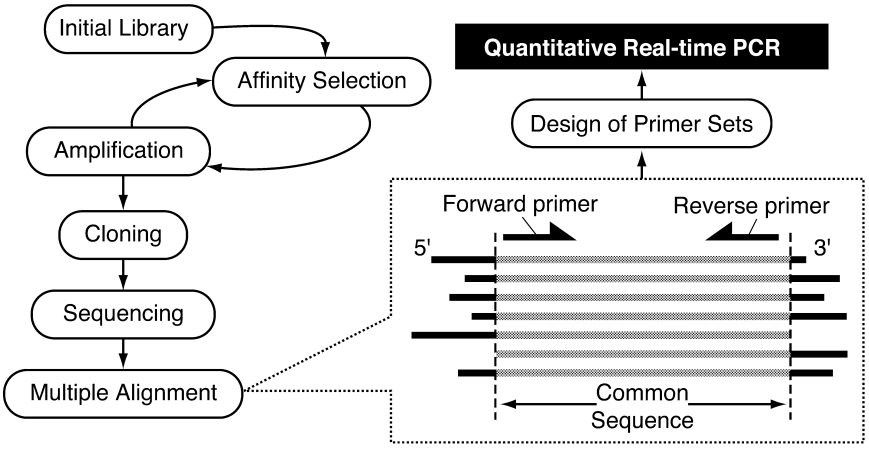

Fig. 1. **A schematic representation of screening with totally *in vitro* display technologies and quantitative real-time PCR analysis.** Initial DNA libraries were constructed by reverse-transcription from poly A (+) RNAs (cDNA library) or chemical synthesis (random-sequence library). Affinity screening and amplification of each library were performed iteratively. After the screening, the resulting library was cloned into a cloning vector and the DNA sequences were determined. The selected DNA sequences were analyzed as described previously (*19*) and were clustered by multiple alignment using the CLUSTALW program (*24*). Primer sets for real-time PCR were designed for each of the clustered sequences. In the case of the sequences of siblings, specific primers were designed based on the common region of the clone encoding each selected sequence. Real-time PCR was performed with a Lightcycler FastStart DNA master SYBR green I kit (Roche) and protein-specific primer sets (the amplicon size was 56 to 200 bp) with a LightCycler (Roche). The standard template DNA was PCR-amplified from each selected sequence on a pDrive vector (Qiagen) using primers 5′M13F (5′-GTTTTCCCAGTCACGACGTTG-3′) and 3′M13R (5′-GAAACAGCTATGACCATGATTACG -3′). The numbers of DNA molecules of the selected sequences in 5 ng aliquots (~10^{10} molecules) of DNA libraries were determined.

confirmed by *in vitro* pull-down assays (data not shown), as had been done with other previously confirmed sequences (*19*). Interestingly, the process of enrichment of each selected clone varied. The highest and lowest enrichment ratios in this selection were about 2.0×10^4 and 80-fold, respectively. Furthermore, the process of enrichment of the clones based on the results of real-time PCR is informative for refining the selection protocol, in particular, the selection conditions.

Figure 3 shows the correlation between the abundance ratio of the 22 selected sequences analyzed by real-time PCR, and that found on cloning and sequencing analysis of the library after the fifth round of screening. The two abundance ratios were well correlated in most cases, but not for the Gas5 and Aes sequences (arrows in Fig. 3). Considering that the two sequences were each discovered as a single clone (Table 1), they are certainly false positives.

Real-time PCR is a PCR application that allows accurate quantification of DNA molecules. This technique is based on the PCR kinetics (*23*); the quantification of amplified DNA molecules is performed during the amplification by using intercalators such as a SYBR Green reagent, and the absolute number of DNA molecules is calculated from the results obtained with standards that are observed at the same time. The real-time PCR technique has been used for gene expression analysis and the detection of mutations. In our previous study, we introduced real-time PCR analysis to evaluate the results of *in vitro* selection. In this paper, we have presented additional data regarding monitoring of the process of enrichment of the selected sequences and elimination of false positives by means of real-time PCR. The use of real-time PCR for this purpose has several advantages over previous methods. For example, binding assays, such as *in vitro* pull-down assays and coimmunoprecipitation, are relatively

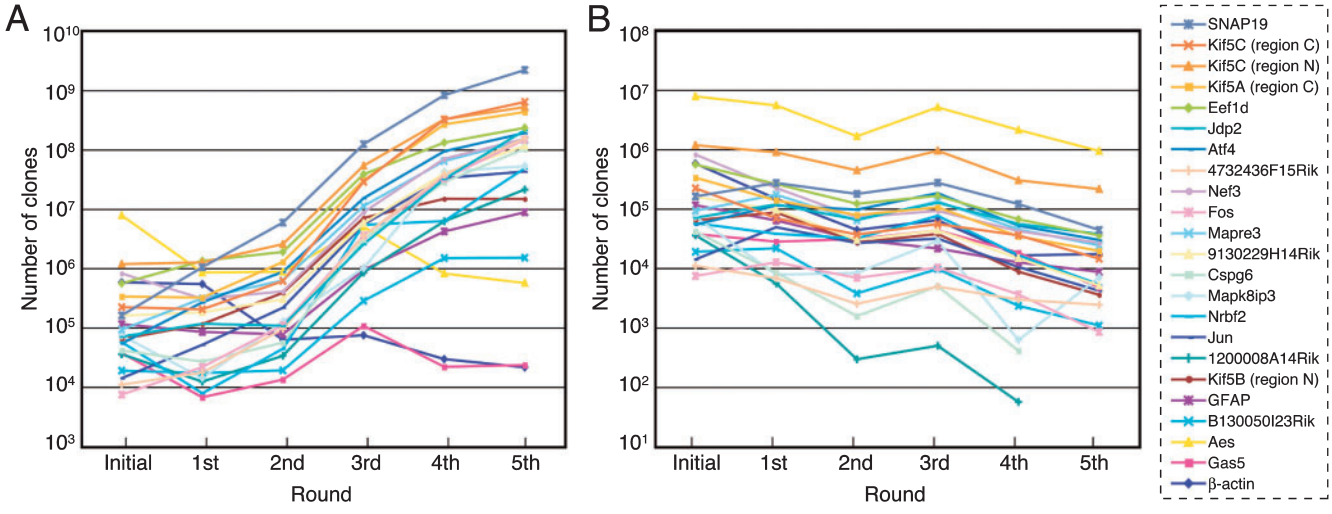

Fig. 2. **Quantitative real-time PCR analysis of the selected sequences in the library obtained on each round.** The numbers of DNA molecules of the 22 specifically selected clones in 5 ng aliquots (~10^{10} molecules) of the libraries, (A) bait (+) and (B) bait (–), obtained on each round were plotted logarithmically. β-Actin was chosen as a negative control.

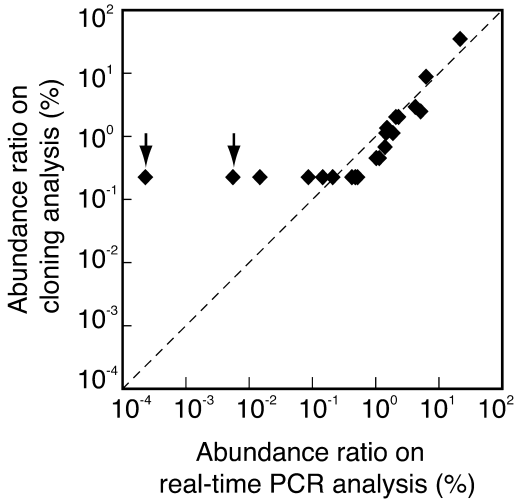

Fig. 3. **Correlation of the abundance ratio on cloning analysis and that on quantitative real-time PCR analysis.** On a logarithmic scale, the vertical axis indicates the abundance ratio on cloning analysis [(number of obtained clones/total number of analyzed clones) × 100 (%)], and the horizontal axis indicates the abundance ratio on real-time PCR analysis [(number of DNA molecules in 5 ng of a DNA library/1.0×10^{10} molecules) × 100 (%)]. The broken line indicates complete correspondence of the two abundance ratios. The arrows indicate Gas5 and Aes from left to right, respectively.

Table 1. **Candidates for Jun-associated proteins selected on IVV screening.**

Gene symbol	Number of clones
SNAP19	155
Kif5C (region C)	39
Kif5A (region C)	13
Kif5C (region N)	11
Eef1d	9
Jdp2	9
Nef3	6
4732436F15Rik	6
Fos	5
Mapre3	3
9130229H14Rik	2
Atf4	2
Cspg6	2
Mapk8ip3	1
Nrbf2	1
Jun	1
1200008A14Rik	1
Kif5B (region N)	1
GFAP	1
B130050I23Rik	1
Aes	1
Gas5	1

tedious to perform. In addition, binding assays involving binary interaction analysis cannot detect indirect interactions, while *in vitro* display technologies can, in principle, detect indirect interactors that form complexes with the bait via direct interactors (*19*).

In summary, we applied real-time PCR to evaluate the sequences of 22 candidate Jun-associated proteins, including three novel candidates picked up on IVV screening. The results show that real-time PCR analysis is a useful tool for monitoring the process of enrichment of specifically selected sequences and for eliminating false positives. This approach should be useful not only for IVV screening, but also for other display technologies, such as ribosome display, DNA display, and phage display.

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