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One-Step Cloning of Murine Fab Gene Fragments Independent of IgH Iso-type for Phage Display Libraries

Phage display of antibody fragments involves cloning and expression of the genes coding for the immunoglobulin light- and heavy-chain regions (8). Generation of murine Fab fragments involves the use of polymerase chain reaction (PCR) primers designed to fit the N-terminal part of the variable light- and heavy-chain domains, the C-terminal part of the light chain and the first constant domain of the heavy chain (CH1) (5). Therefore, the isotypes of the variable heavy-chain gene (VH) repertoire are determined by the choice of the constant-domain PCR primers. So far, primers corresponding to the genes of the gamma isotype have been used exclusively (6,9), because the mature immune response in mice is dominated by the IgG isotype. Yet, B-cell antigens, such as carbohydrates, which do not stimulate T cells, are

Figure 1. General outline of VH PCR assembly procedure (top) and agarose gel electrophoresis of DNA fragments generated in the various assembly reactions (bottom). Arrows represent primers; boxes represent PCR-amplified DNA fragments. VH = Heavy-chain-variable region gene; LC = light chain gene; CH1 = the first constant domain of the heavy-chain IgG1 isotype; LINK-D = linker DNA fragment.
known to induce antibodies of the IgM isotype primarily. Therefore, it is not always possible to predict which isotype will be chosen by the immune system. Inspection of available B-cell hybridomas of murine origin reveals that many useful antibodies have isotypes (mostly IgM and IgA) that are not normally included in the PCR primers used for the cloning of antibody Fab fragment genes.

One approach to obtaining antibodies that correspond to all isotypes is to clone the variable regions only and create single-chain Fv (scFv) fragments (7). Unfortunately, these fragments are less stable and usually have lower antigen-binding affinity than the corresponding Fab fragments (2). scFv fragments would therefore not fully represent the repertoire of V genes obtained from immunized mice. Another approach is to design primers matching the CH1 region of all known isotypes. This approach has potential disadvantages because the successful expression of murine Fab fragments in Escherichia coli has been reported to be isotype-dependent (1). Thus, certain Ig isotypes could be favored during the expression of the library, and this would lead to a biased VH-gene repertoire.

To circumvent these problems, we have developed a procedure for cloning and expressing murine Fab fragments independent of the IgH isotype chosen by the immune system. This procedure involves amplification of the VH part of the heavy-chain gene and its linkage to a fragment coding for the first domain of the IgG1 isotype (CH1γ1), thereby converting it into an Fd gene (the segment that codes for the heavy chain of the Fab fragment). Finally, the Fd gene fragments are joined to the light-chain gene fragments (LC). An overview of our PCR assembly procedure is shown in Figure 1.

Our procedure requires the construction of a CH1-LINK-D fragment, which contains the CH1γ1 gene fused to the linker fragment LINK-D and the use of a set of primers complementary to the J sequences of the heavy chain. These primers, and the primers used for the PCR amplification of the CH1-LINK-D, are shown in Figure 2.

Using these tools and primers described in Reference 9, we generated a library from immunized mice. The VH and LC genes were amplified in two separate reactions (Figure 1, lanes 1 and 5) using each reaction 0.2 μM dNTP, reaction buffer supplied by the manufacturer (Perkin-Elmer, Norwalk, CT, USA), primers (VH: 50 pmol of MVH 1-25 and 50 pmol MJH 1-4; LC: 50 pmol MVK 1-25 and 50 pmol MCK) and template cDNA made from 30 μg of total RNA isolated from spleens of immunized mice (3). The reaction mixtures (100 μL) were overlaid with paraffin oil and heated to 94°C for 5 min before adding 1.5 U AmpliTaq® (Perkin-Elmer) per reaction. The mixtures were cycled 30 times (94°C for 1 min, 55°C for 1 min, 72°C for 1 min), followed by incubation at 72°C for 10 min. The primary PCR products were gel-purified and used as templates in PCR assembly reactions where the VH genes were joined with CH1-LINK-D and the LC genes with LINK-D (Figure 1, lanes 2 and 4). The two linker DNA fragments were obtained by PCR amplifications using templates, which contained the appropriate linker sequences (9). The 100-μL reactions contained dNTP and buffer as above. The VH Linker PCR assembly reaction contained 10 ng VH fragment (about 1/20 of the primary PCR product), 50 pmol CH1-LINK-D fragment and primers (20 pmol TAGBACK and 20 pmol LINKFORW). The LC Linker PCR assembly reaction contained 10 ng LC fragment (about 1/20 of the primary PCR product), 1 ng LINK-D fragment and primers (20 pmol TAGFORW and 20 pmol LINKBACK). The reaction mixtures were treated as above and cycled 25 times (94°C for 1.5 min, 65°C for 1 min, 72°C for 1.5 min), followed by incubation at 72°C for 10 min and gel-purification of the amplified products. The final PCR assembly reaction (Figure 1, lane 3) contained dNTP and buffer as before in a 100-μL reaction volume that included 5 ng VH/CH1-LINK-D, 5 ng LC/LINK-D and 10 pmol ASSEMBLY primer. The reaction mixture was hot-started as above and cycled 25 times (94°C for 2 min, 72°C for 2 min), followed by incubation at 72°C for 10 min. The final PCR product was gel-purified, digested with SfiI and NotI (3) and cloned into the expression vector pFab5c (9). The resulting library consisted of about 107 independent clones. Use of extensive rounds of PCR amplification increases the risk of introducing mutations that might lead to libraries dominated by nonfunctional genes. To examine the functionality of our library, we analyzed 20 randomly picked clones prior to the bio-panning procedures and found 80% of the clones to be Fab producers (data not shown). By subsequent panning of the library, we isolated antigen-specific clones with high specificity and affinity (unpublished).

We have noted that the successful VH/CH1-LINK-D PCR assembly requires a larger amount of template than

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**Mouse J region primers (anti-sense)**

| MJH-1    | GGG GGT GTC GTT TTG GCT GAG GAC AGC GTG ACC GTG G |
| MJH-2    | GGG GGT GTC GTT TTG GCT GAG GAG ACT GTG AGA GTG G |
| MJH-3    | GGG GGT GTC GTT TTG GCT GCA GAG ACA GTG ACC AGA G |
| MJH-4    | GGG GGT GTC GTT TTG GCT GAG GAC AGC GTG ACT GAC G |

**New primer for amplification of linker DNA**

| GTC TCT KCA GCC AAA AGC ACA CCC CC |

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**Figure 2. Primers for PCR amplification and assembly of the VH gene.**
the LC/LINK-D assembly. The sluggishness of the VH/CH1-LINK-D reaction may be caused by formation of strong secondary structures within the coding region of CH1, which would also account for the relatively weaker amplification of the Fd gene (as compared to the VH gene) as observed previously (9).

Although the PCR assembly strategy has been described as somewhat difficult (4), we find it an attractive alternative to the cloning of heavy- and light-chain genes separately. Furthermore, the rare cutting-restriction enzymes SfiI and NotI are the only enzymes used in the entire cloning procedure. Use of more enzymes inevitably increases the danger of cleavage within the Fab coding regions, which would lead to less comprehensive libraries.

REFERENCES


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Peter Sejer Andersen, Henrik Ørum and Jan Engberg

The Royal Danish School of Pharmacy

Copenhagen, Denmark

Sensitive, Nonradioactive Differential Display Method Using Chemiluminescent Detection

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Isolation of genes that are differentially expressed in closely related but different cell types, such as normal and cancer cells, is of great interest in modern molecular biology. The classical approaches that have been used widely for isolation of differentially expressed genes are subtractive hybridization and differential hybridization (7). Although many successful applications have been reported in different systems, these methods are both time-consuming and technically difficult. The recently described mRNA differential display method provides an attractive, alternative tool for cloning differentially expressed genes and has gained considerable attention (3). In the differential display method, mRNAs from two or more cell populations are reverse-transcribed and amplified by polymerase chain reactions (PCR) using one 3' anchored primer and one 5' arbitrary primer. The radioactive cDNA products from PCR are then resolved on a DNA sequencing gel in adjacent lanes. Differentially expressed mRNA species are identified by comparing the intensities of cDNA bands on the gel.

Currently, radioactive detection methods are used by most researchers to identify band patterns on a sequencing gel in a differential display experiment (3,4). The disadvantages of the standard procedure include the handling of hazardous radioisotopes, potential contamination of the working area and thermal cycler, and cost and burden of waste disposal of the radioactive materials. Recently, a silver staining-based differential display protocol has been described (5). Although valuable, this method has some drawbacks. The detection sensitivity by silver staining is generally much lower than that obtained by using radioisotopes. This limitation leads to much fewer bands being displayed per reaction (5) and causes low-abundance messages to be undetectable. Furthermore, silver-