# Chapter 2

# Analysis of Transcription Factor Networks Using IVV Method

**Hiroyuki Ohashi, Shigeo Fujimori, Naoya Hirai, Hiroshi Yanagawa, and Etsuko Miyamoto-Sato**

## Abstract

We have developed a simple and totally in vitro selection procedure based on cell-free cotranslation using a highly stable and efficient in vitro virus (IVV). Cell-free cotranslation of tagged bait and prey proteins is advantageous for the formation of protein complexes and allows high-throughput analysis of protein–protein interactions (PPI) as a result of providing in vitro instead of in vivo preparation of bait proteins. The use of plural selection rounds and a two-step purification of the IVV selection, followed by in vitro post-selection, is advantageous for decreasing false positives. This simple IVV selection system based on cell-free cotranslation is applicable to high-throughput and comprehensive analysis of transcription factor networks.

**Key words** Cell-free protein synthesis system, In vitro selection, Transcription factor, mRNA display, PPI

## 1 Introduction

The identification of PPI networks is an important aspect of proteomics research. In vitro selection experiments using mRNA display methods, which were originally developed for evolutionary protein engineering, such as in vitro virus (IVV, Fig. 1) [1–3] or mRNA–peptide fusions [4–6], are powerful tools for the analysis of protein function [7, 8].

Basically, mRNA display is composed of four essential processes, i.e., transcription, translation, selection, and reverse transcription-polymerase chain reaction (RT-PCR). Our newly developed cell-free cotranslation technique provides a totally in vitro manipulation that is suitable for high-throughput, genome-wide analysis as a result of in vitro bait translation instead of in vivo bait preparation. Cotranslation of bait and prey proteins should also be advantageous for the formation of protein complexes [9].

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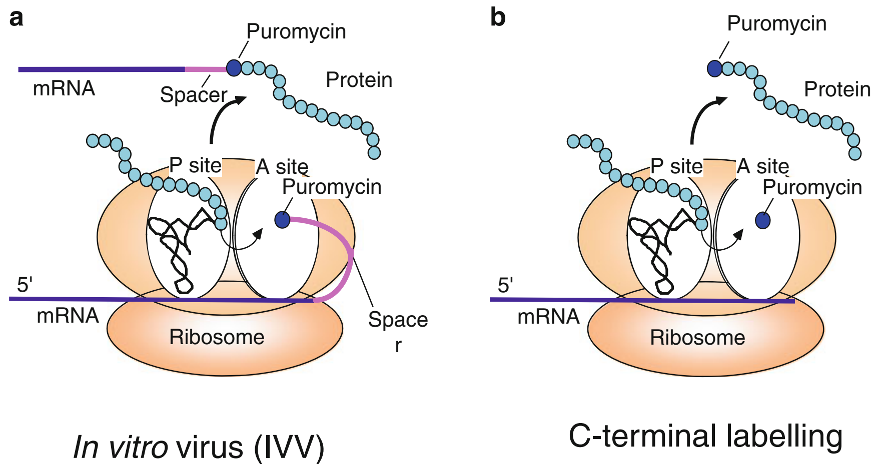


**Fig. 1** Puromycin technology; the C-terminal protein labeling and IVV methods. (**a**) IVV formation on the ribosome. Puromycin at the 3′-terminal end of a spacer (Fluor-PEG Puro) ligated to mRNA can enter the ribosomal A-site to bind covalently to the C-terminal end of the encoded full-length protein in the ribosomal P-site. (**b**) C-Terminal labeling of proteins on the ribosome. A puromycin derivative (Fluor-dCpuro) can enter the ribosomal A-site to bind covalently to the C-terminal end of the protein in the ribosomal P-site

This approach would offer a good chance to obtain a comprehensive data set including not only direct but also indirect interactions in a single experiment.

Decreasing false positives is important to obtain data that are, at least potentially, biologically relevant. Accordingly, we used two-step purification of the IVV selection based on the TAP method [10], which is suitable for analysis of protein complexes formed by capture from a crude mixture with a tagged bait protein, with a low level of false positives. To further decrease false positives and to obtain information about direct/indirect interactions, an in vitro post-selection was performed.

The post-selection is composed of a pull-down assay to confirm the interactions using C-terminal protein labeling [2, 3, 11, 12] and real-time polymerase chain reaction (PCR) assay to confirm the enrichments. The use of two-step purification of the IVV selection followed by post-selection should provide reliable data for PPI analysis.

Here, we present a simple and totally in vitro IVV selection method based on cotranslation of bait and prey proteins using tagged protein and a prey cDNA library. After IVV selection, we perform sequence analysis, and the sequence data were analyzed computationally to assign genes to rapidly generate a PPI map. An in vitro post-selection was carried out to confirm positive interactions before PPI mapping and in silico analysis.

## 2 Material

### *2.1 Preparation of a cDNA Library and Bait Protein*

1. Random priming reverse transcription (RT) and preparation of dsDNA kit; SuperScriptII Double Strand cDNA Synthesis Kit (Invitrogen).
2. mRNA synthesis kit; RiboMAX Large Scale RNA Production System-SP6 (Promega).
3. RNA purification kit; RNeasy MinElute Cleanup Kit (Qiagen).
4. PEG-Puro spacer; p(dCp)$_2$-T(Fluor)p-PEGp-(dCp)$_2$-puromycin (Fig. 2).
5. T4 RNA ligase (Takara, Otsu, Japan).

### *2.2 In Vitro Selection*

1. In vitro translation kit; Wheat germ extract reaction mixture (Promega).
2. RNase inhibitor (Invitrogen).
3. Beads for purification of IVV; Rabbit immunoglobulin G (IgG) agarose beads (Sigma).
4. IPP150 buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.1 % NP-40).

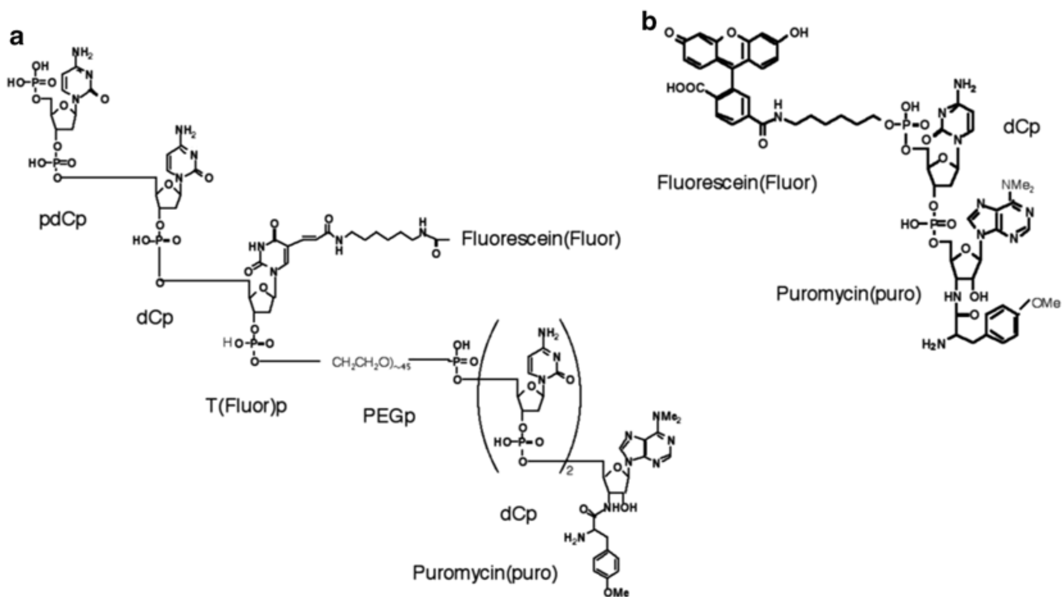


**Fig. 2** PEG puromycin spacer for the IVV method and puromycin analogues for the C-terminal protein labeling method. (**a**) The structure of PEG puromycin spacer. Fluor-PEG Puro spacer [p(dCp)2-T(Fluor)p-PEGp-(dCp)2-puromycin] was synthesized from Puro(Fmoc)-CPG, Polyethylene glycol (PEG, average mol. wt 2,000), deoxycytidine phosphoramidite (dC-amidite), thymidine(fluorescein) phosphoramidite [T(Fluor)-amidite]. (**b**) The structure of fluorescence-labeled puromycin. A fluorophore (herein, fluorescein) was chemically joined to puromycin through a dC linker

5. TEV cleavage buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1 % NP-40, 0.5 mM EDTA, and 1 mM DTT).
6. TEV protease.
7. OneStep RT-PCR kit (Qiagen).
8. PCR cloning kit (Qiagen).

## 3 Methods

### *3.1 Preparation of a cDNA (Prey) Library from Cells (See Fig. 3 and Note 1)*

1. Perform random priming reverse transcription (RT) and preparation of dsDNA using the SuperScriptII Double Strand cDNA Synthesis Kit (Invitrogen).
2. Perform RT using a poly(A)+ mRNA library with a primer (TCATCGTCCTTGTAGTCAAGCTTNNNNNNNNN).
3. Perform ligation reaction of a synthetic double-stranded adaptor (GGAATTCG and GAACAACAACAACAACAAACAACAAC AAAATGGCTAGCATGACTGGTGGACAGCAAATGGCG AATTCC) to the 5′ end of the dsDNA.
4. Amplify the adaptor-ligated cDNA library using PCR with 5′IVVlib (GGAAGATCTATTTAGGTGACACTATAGAACAA CAACAACAACAAACAACAACAAAATG) and 3′IVVlib (TTT TTTTTCTTGTCGTCATCGTCCTTGTAGTCAAGC).
5. Perform transcription of the cDNA library and ligation with the PEG Puro spacer (Fig. 2) using the RiboMAX Large Scale RNA Production System-SP6 (Promega) and m7G(5′)ppp(5′) G RNA Capping Analog (Invitrogen Corp., Carlsbad, CA, USA).
6. The resulting RNA should be purified with the RNeasy mini kit and ligated with a PEG-Puro spacer p(dCp)$_2$-T(Fluor) p-PEGp-(dCp)$_2$-puromycin (Fig. 2) using T4 RNA ligase (Takara, Otsu, Japan).

Bait

| SP6 | O' | T7 tag | cDNA | CBP | ZZ domain | His Tag |
|---|---|---|---|---|---|---|

ATG TEV

Prey

| SP6 | O' | T7 tag | cDNA | Flag tag | Poly A-Tail |
|---|---|---|---|---|---|

ATG

**Fig. 3** The sequence contains a SP6 promoter, a part of the omega sequence named O′ [13], an N-terminal T7-tag coding sequence, and a C-terminal affinity tag, the coding sequence for the IgG binding domain of protein A (ZZ domain), a TEV protease cleavage site, and a calmodulin binding peptide

7. Purify the ligated RNA with the RNeasy mini kit and in vitro-translated in the wheat germ cell-free translation system to create the mRNA-displayed peptide library.

### 3.2 Preparation of the Bait RNA Template

1. Template DNA for a bait protein; an example of a template DNA construct for a bait protein is shown in Fig. 3. The sequence contains a SP6 promoter, a part of the omega sequence named O′ [13], an N-terminal T7-tag coding sequence, and a C-terminal affinity tag, the coding sequence for the IgG binding domain of protein A (ZZ domain), a TEV protease cleavage site, and a calmodulin binding peptide.
2. Prepare the DNA template of a bait protein using PCR with Ex Taq (Takara Bio).
3. Prepare mRNA using mRNA synthesis kit; RiboMAX Large Scale RNA Production System-SP6 (Promega) and m7G(5′)ppp(5′)G RNA Capping Analog (Invitrogen).
4. Purify mRNA with a RNA purification kit; RNeasy MinElute Cleanup Kit (Qiagen).

### 3.3 In Vitro Selection

1. Incubate 50 μl aliquot of wheat germ extract reaction mixture (Promega) containing 10 pmol of the bait RNA, 10 pmol of the ligated library RNA, 80 μM amino acid mixture, 76 mM potassium acetate, and 40 U of RNase inhibitor (Invitrogen) for 1 h at 26 °C (*see* **Note 2**).
2. Add the reaction mixture to 50 μl of rabbit immunoglobulin G (IgG) agarose beads (Sigma) equilibrated with 50 μl of IPP150 buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.1 % NP-40), and mix on a rotator for 2 h at 4 °C (*see* **Note 3**).
3. Wash the beads with 800 μl of IPP150 buffer once and with 800 μl of TEV cleavage buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, and 1 mM DTT) five times.
4. Add 100 μl of TEV cleavage buffer containing 100 U of TEV protease. Rotate for 2 h at 16 °C.
5. The resulting elution can be used as the RT-PCR template.

### 3.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Sequencing of Selected Clones

1. Perform RT-PCR with OneStep RT-PCR kit (Qiagen, *see* **Note 4**) using primers, 5′F3 (5′-GGAAGATCTATTTAGGTGACACTATAGAACAACAACAACAACAAACAACAACAAAATG-3′) and 3′Flag-1AL (5′-TTTTTTTTCTTGTCGTCATCGTCCTTGTAG-3′).
2. The optimal number of PCR cycles without reaching a plateau is 26–30 cycles at each RT-PCR step. The RT-PCR product was used for the next round of selection (*see* **Notes 5** and **6**).
3. After five rounds of affinity screening, the RT-PCR product can be cloned using a PCR cloning kit (Qiagen) and sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

***3.5 Sequence Analysis***

1. Selected clones are subjected to nucleotide–nucleotide BLAST (BLASTN) search to identify the protein represented by each clone.
2. The nucleotide database obtained from the NCBI ftp site (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) can be employed as a reference database.

***3.6 Pull-Down Assay***

1. Perform pull-down assay using the C-terminal fluorescence labeling technique to confirm the predicted PPIs.
2. Amplify the DNA templates from the cloned plasmids with primers, 5′F3 and 3′R3 (5′-TTTTTTTTCTCGAGCTTGT CGTCATCG-3′).
3. Use the amplicons as templates for transcription. Perform a translation reaction with the resulting mRNAs in the presence of fluorescence-labeled puromycin to make fluorescence-labeled proteins (Fig. 2).
4. Perform a translation reaction of bait protein in the cell-free translation system separately.
5. Mix these translated proteins together and incubate with rabbit IgG agarose beads.
6. Elute the binding proteins with sample buffer (0.1 M Tris–HCl, pH 6.8, 4 % SDS, 0.2 % bromophenol blue, and 20 % glycerol) at 100 °C for 5 min, subjected to 17.5 % SDS-PAGE, and analyzed with a Molecular Imager FX (Bio-Rad Laboratories).
7. An example of a PPI map is shown in Fig. 4.

## 4 Notes

1. The template DNA requires a SP6 promoter sequence and O′ sequence upstream of the open reading frame (ORF). ORF must initiate with an initiation codon (ATG).
2. For formation of the 1-h incubation is sufficient. Longer incubation may destabilize the IVV complex.
3. Because the mRNA is unstable in the cell-extract system, the reaction mixture must be placed at 4 °C after translation reaction.
4. Two-step RT-PCR can be also performed.
5. It is recommended that a high-fidelity DNA polymerase be used to predict correct sequences from the selected peptides and to minimize the effects of errors with a polymerase after selection.
6. If the diffuse band or by-products are identified on an agarose gel electrophoresis, the product with expected length is purified from a gel by MinElute Gel purification kit (Qiagen).

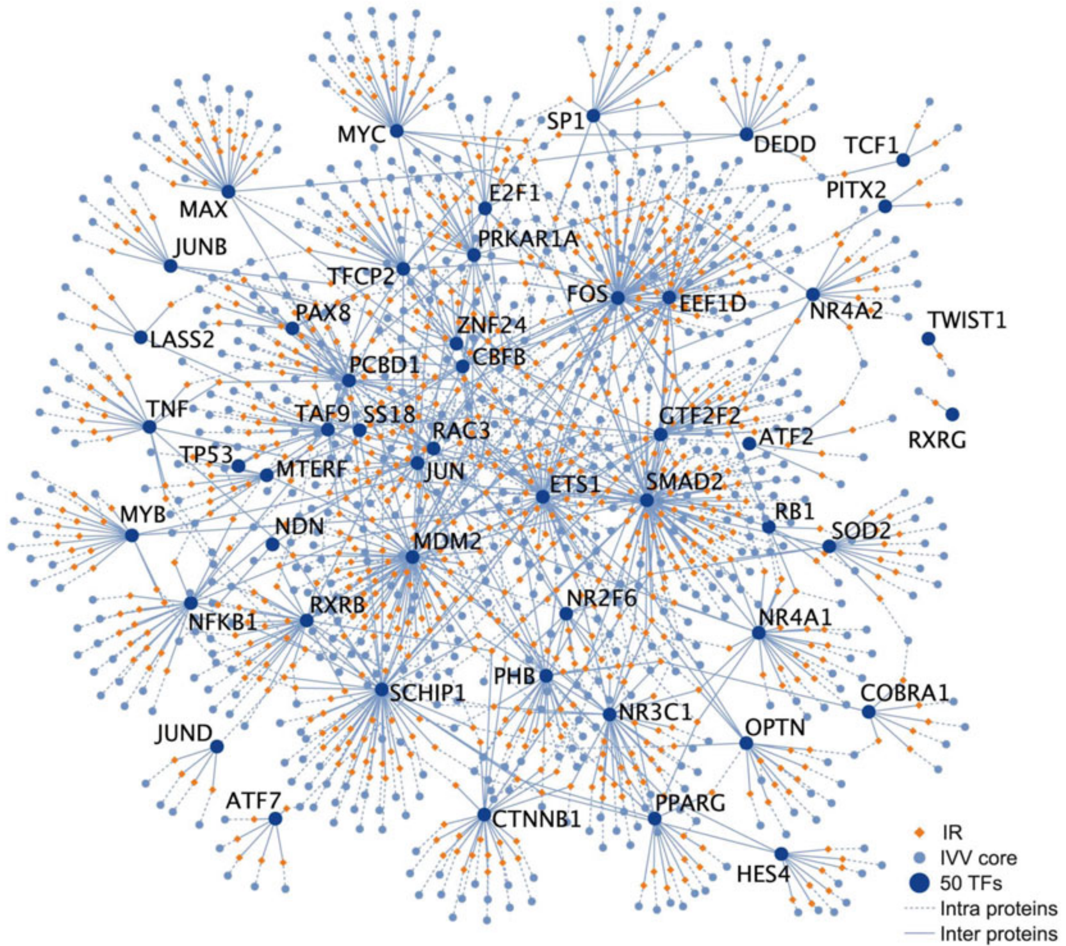


**Fig. 4** A transcription factor network at the interaction region (IR) level developed using IVV data. Graphic expression of the PPI network at the IR level. Interacting interfaces of the proteins, determined as IRs by IVV experiments, are drawn on the graph as *diamond-shape nodes* (IR nodes). *Broken* and *solid lines* indicate "intra-" and "inter-" protein edges, respectively. The graph contains 1,572 nodes (842 IR nodes and 730 protein nodes) and 842 intra-protein edges. Note that overlapping IRs are merged into a single node in the constructed network

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