CellFree Sciences

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High Performance Cell-Free Wheat Germ Protein Expression System

Application Note

Wheat germ cell-free protein expression enables studies on E3 ubiquitin ligases

Abstract

With about 600 to 700 E3 ubiquitin ligases encoded within the human genome, it becomes a complex task to map out the substrate-E3 ligase interactions underlying the regulation of protein degradation in the cell. The wheat germ cell-free protein expression system from CFS, however, provides suitable means for preparing E3 ligases on a high throughout. This allowed different groups to do protein-protein interaction screening experiments and to identify binding partners for E3 ligases.

Introduction

Protein degradation is tightly controlled in the cell, and thus closely relates to physiological conditions and disease [1, 2]. The ubiquitin proteasome system uses ubiquitination to specially mark proteins for degradation or altering protein functions. Protein ubiquitination is triggered by an enzymatic cascade involving a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and an E3 ubiquitin ligase [1]. With ~5 % of all genes encoding E3 ligases, they make up for one of the largest gene family in the human genome. The substrate specificity of those about 600 to 700 human E3 ligases by large controls the regulatory network behind ubiquitination. While this complexity of the E3 ligases offers great flexibility for cellular responses, it also offers great challenges to researchers to identify the substrates for E3 ligases leaving as out of today many enzymes uncharacterized [3]. However, effective means for studying E3 ligase-substrate interactions are of crucial importance to utilize E3 ligases as possible drug targets, e.g. for cancer therapy [4, 5] or neurological disorders [6]. Here, we provide some examples on how the wheat germ cell-free protein expression system was successfully used to study E3 ligase-substrate relationships in high-throughput experiments.

Making proteins on high throughput

Cell-free protein expression can be automated to allow for parallel expression of many proteins [7]. Like in the case of E3 ligases, these experiments often start from cDNA clones available from large genome projects [8]. To avoid sub-cloning of the open reading frames from the original cDNA clones into an expression vector for the wheat germ system, large-scale experiments commonly prepare expression templates by PCR. The PCR products obtained from the cDNA clones can be directly used in the cell-free protein expression system, where overlap extension PCR provides flexible means to introduce additional sequences at the 5’ and 3’ end of the template. Such added sequences can provide the RNA polymerase promoter (here SP6), a translation enhancer (here E01), and possibly affinity tags among other features. For the wheat germ system, a secure “Split-Primer” protocol was developed for preparing expression templates by PCR (Figure 1). The name refers to the

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need of two "Split-Primers" to reconstitute an active SP6 promoter [9]. This reduces the risk that miss-primed PCR products could possibly yield undesired proteins in the later translation reaction.

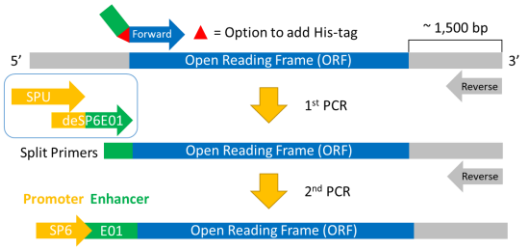

Figure 1: Outline of "Split-Primer" PCR to prepare templates for cell-free protein expression. A 3' overhang is added to the ORF for stabilizing the linear DNA and RNA.

PCR reactions can be automated and performed on multiwell plates. The same multiwell plate format can afterwards be used to setup the RNA transcription and protein expression reactions. To improve the protein yields of the small translation reactions, CFS uses a special "bilayer" format (Figure 2) that allows to maintain the translation reaction for up to 20 hours thus exceeding the yields of regular batch reactions [10]. Combining PCR template preparation with automated protein expression enables genome-wide protein expression experiments, e.g. for use in protein array preparation or proteomics.

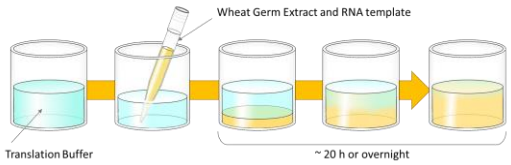

Figure 2: Outline of "Bilayer" translation reaction. During reaction setup, the wheat germ extract with the RNA template is placed below the translation buffer. Both layers mix over time for a constant supply of the protein expression reaction.

Working with biotinylated proteins

Working with proteins in screening experiments often requires their immobilization, e.g. by binding to beads for the detection reaction. The very strong non-covalent binding of biotin to streptavidin is widely used in such analytical assays. Adding the *E. coli* biotin ligase BirA to wheat germ cell-free protein expression reactions allows for specific mono-biotinylation of proteins having a short recognition sequence for the BirA ligase common added to the N-terminus [11]. This approach was utilized to develop screening assays for the PerkinElmer AlphaScreen™ (Amplified Luminescent Proximity Homogeneous Assay) system (Figure 3). Since the labeling reaction is very specific, crude protein expression reaction mixtures can be directly used in the AlphaScreen™ assays omitting any needs for protein purification. Such AlphaScreen™ assays in combination with the wheat germ system have been used, for example, to screen serum samples for autoimmune antibodies.

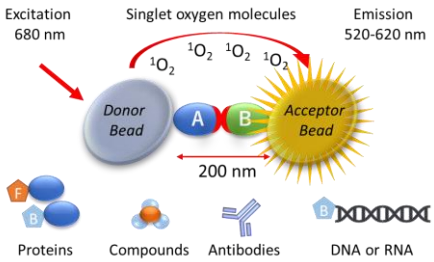

Figure 3: Outline of the AlphaScreen™ assay for the detection of interactions between two binding partners. The system was used to study protein-protein interactions with E3 ligases, but the assay can also be used to study binding to chemical compounds, DNA, or RNA molecules.

Studies on E3 ligases

The scalability of the wheat germ cell-free protein expression system was used in several projects to analyze E3 ligases. Ramadan et al. [12] used the RIKEN Arabidopsis full-length cDNA library in combination with PCR-driven template preparation to make protein libraries of Arabidopsis E2 and RING E3 enzymes. In total 35 E2 ubiquitin conjugating enzymes and 204 RING-type E3 ubiquitin ligases were prepared for functional characterization. Thioester assays using dithiothreitol (DTT) showed DTT-sensitive ubiquitin thioester formation for all expressed E2 enzymes. Similarly, all the 27 tested RING E3 ligases showed ubiquitin ligase activity,

indicating that the wheat germ expression system is a suitable tool for studying the ubiquitination system.

In another study, a protein array containing 227 human and 23 mouse E3 ligases was prepared by the wheat germ system and analyzed in high-throughput binding experiments again using the AlphaScreen™ method [13]. To proof the concept, the binding of p53 and MDM2 proteins to E3 ligases within the protein array was tested, which identified interactions with 11 E3 ligases including four enzymes known to target p53. Thus, the authors concluded that their approach is suitable for screening substrate-E3 ligase interactions.

The approach was later used again by the same group to study the regulation of the transcription factor RUNX1 and its role in hematopoiesis and leukemia. RUNX1 is regulated by proteolytic degradation through the ubiquitin-proteasome pathway, but it was unknown which E3 ligases are in control of its ubiquitination. Screening a set of 287 E3 ligases prepared in the wheat germ system, it was found that E3 ligase STUB1 bound to RUNX1 and induced its ubiquitination and degradation mainly in the nucleus [14]. STUB1 also induced the ubiquitination of the related leukemogenic fusion protein RUNX1-RUNX1T1. Overexpression of STUB1 induced growth-inhibition in myeloid leukemia cells expressing RUNX1-RUNX1T1. This observation points at the importance of E3 ligases as targets for cancer therapy, where possibly activation of STUB1 could be used to target RUNX1-RUNX1T1-dependent leukemia.

A different approach had been taken by Kasahara K. et al. [15] to study the control of ciliogenesis during axoneme extension. They used the wheat germ system to prepare in total 1,172 E3 ligase proteins (including putative E3 enzymes) from the human proteome expression resource library (HuPEX) [16]. To identify E3 ligases involve in trichoplein polyubiquitylation and ciliogenesis, a special protein array platform (the “Protein Active Array”, Figure 4) was developed and then used to screen their global E3 ligase set in a two-step process. This screen led to the identification of KCTD17 as a substrate for E3 ligase CRL3s. CRL3s polyubiquitylates trichoplein and its degradation initiates axoneme extension during ciliogenesis.

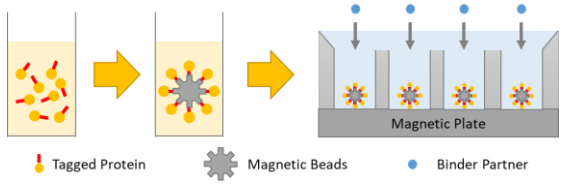

Figure 4: Outline of “Protein Active Array” format: GST-tagged proteins are expressed in the wheat germ system. After completion of the translation reaction, glutathione-coated magnetic beads are added to capture and purify the GST-tagged proteins. The magnetic beads along with the bond proteins are then transferred into dedicated magnetic plates that hold the beads at the bottom of the wells. Immobilized proteins in the wells are used as baits during protein-protein interaction screens.

Conclusion

The important role of the ubiquitin protease system to maintain most cellular processes makes it an important target for research and drug discovery [17]. Within this system, the substrate specificity of E3 ligases often controls which proteins are selected for modifications. Hence, E3 ligase inhibitors are actively studied and disrupting substrate-E3 ligase protein-protein interactions could be one way to search for new lead molecules. The human E3 ligase sets described in the publications mentioned here could be valuable tools to facilitate such screens.

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