 doi:10.20944/preprints202107.0531.v1

HALO-RPD: In Searching for RNA-Binding Protein Targets in Plants

A.O. Shamustakimova

All-Russian Research Institute of Agricultural Biotechnology, Timiryazevskaya st., 42, Moscow 127550 Russia

E-mail: nastja_sham@mail.ru

Abstract

Study of RNA-protein interactions and identification of RNA targets are among the key aspects of understanding the RNA biology. Currently, various methods are available to investigate these interactions, in particular, RNA pulldown assay. In the present paper, a method based on the HaloTag technology is presented that is called Halo-RPD (**Halo**Tag **R**NA **P**ull**D**own). The proposed protocol uses plants with stable fusion protein expression and the MagneBeads magnetic beads to capture RNA-protein complexes directly from the cytoplasmic lysate of transgenic *A. thaliana* plants. The key stages described in the paper are as follows: 1) preparation of the magnetic beads 2) tissue homogenization and collection of control samples 3) precipitation and wash of RNA-protein complexes; 4) evaluation of protein binding efficacy; 5) RNA isolation; 6) analysis of the obtained RNA. Recommendations for better NGS assay designs are provided.

Keywords: *A.thaliana* - HaloTag – RNA-binding proteins – RNA pulldown assay – RNA-protein complexes – cold shock domain protein

INTRODUCTION

RNA-binding proteins play a major part in complex cellular processes, such as differentiation, development, responses to biotic and abiotic stress factors, and post-transcriptional control.

In recent years, the variety of methods to study RNA-protein interactions has expanded significantly [1]. However, some older technologies, such as RNA-immunoprecipitation (RIP), still remain rather common [2]. The latter is based on *in vivo* mapping of RNA-protein interactions using crosslinking agents, such as ultraviolet or formaldehyde. Currently, RIP is used in a vast majority of studies investigating RNA-protein complexes in plants [3-6].

Despite its wide use, RIP has several downsides, e.g. UV radiation induces the formation of the irreversible covalent bond between a protein and RNA; formaldehyde not only binds the protein of interest to RNA but crosslinks its partner proteins as well; highly specific antibodies are required for a successful outcome.

HaloTag-fused proteins are used in the proposed protocol as an alternative to RIP [7]. Initially, the HaloTag technology was intended and successfully used for precipitation of protein-protein and DNA-protein complexes from bacterial and mammalian cell lysate [8]. However, in the last few years, the technology was adapted [9-11] and modified [12] to identify RNA-protein complexes in tissue cells in humans and animals.

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So far, there have been only two papers, whose authors investigated the use of HaloTag technology for the search of partner proteins in plants [13, 14]. In the first paper, the authors analyzed mediator proteins in transgenic plants of rice, and in the second one, the binding site of transcription factor *Zm*NST3 in transgenic plants of maize was investigated.

The goal of the presented study was to design a protocol for isolating RNA-protein complexes from cytoplasm of *Arabidopsis thaliana* plants using the HaloTag technology.

Papers [10] and [15] were used as a reference to design the protocol called **Halo**Tag **R**NA-**P**ull**D**own (Halo-RPD).

THE HALO-RPD METHOD

Plant material. Two lines of *Arabidopsis thaliana* (L.) Heynh. Columbia ecotype were used in the study as transgenic plants with stable expression of HaloTag and *Es*CSDP3-HaloTag-fused proteins [16]

The stages of HaloRPD protocol for isolation of RNA-protein complexes are as follows: preparation of magnetic beads; tissue homogenization, and collection of control samples; pull-down and wash of RNA-protein complexes; evaluation of protein binding efficacy; RNA isolation; analysis of the obtained RNA. A simplified assay design is presented in Fig. 1.

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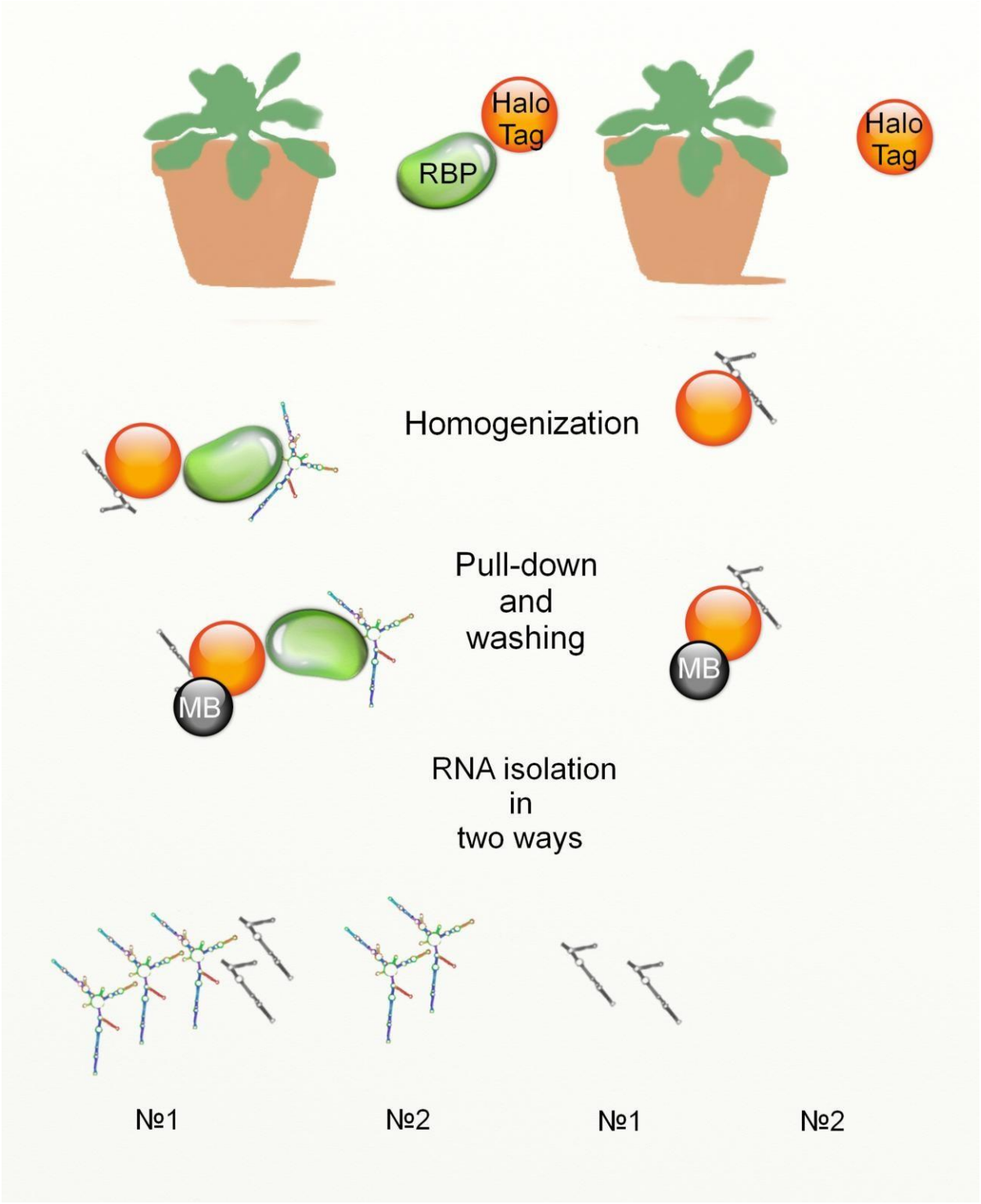

Fig. 1. Simplified Halo-RPD assay design. Transgenic plants expressing HaloTag-fused RNA-binding protein (on the left) and the plants expressing the HaloTag protein (on the right). Plant tissue is homogenized to obtain cytoplasmic lysate and incubated with the magnetic beads capable of binding HaloTag protein by covalent bonds. The beads with precipitated target protein complexes are washed, and RNA is isolated in two ways. The first way is to isolate RNA directly from the magnetic beads by incubation in the ExtractRNA reagent, and the second implies elution in TEV buffer and further isolation using the ExtractRNA reagent.

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Preparation of magnetic beads

The Magne Halotag Beads (Promega Corp.) magnetic particle suspension (100µl) was placed in two tubes for the experimental (*Es*CSDP3-HaloTag) and control (HaloTag) samples. The tubes were incubated on a magnetic stand until transparent, and the liquid was carefully removed without disturbing the beads. The buffer (400 µl) composed of 50 mM Tris - HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Igepal Ca-630 (Promega Corp.) was added to the beads, and the suspension was gently mixed manually several times. The tubes were transferred to a magnetic stand, incubated until transparent, and liquid was fully removed by pipetting. The tubes were then washed two more times. The supernatant was not removed after the third wash. The tubes were stored at 4 ºC.

The manufacturer offers two types of substrate for pull-down of fusion protein from lysate, namely HaloLink resin (Promega Corp.) and MagneBeads (Promega Corp.), the latter being a newer product. The magnetic beads have an advantage of high binding affinity of HaloTag-fused proteins and low non-specific binding level. For instance, 1 ml of magnetic beads binds over 20 mg of protein, whereas the same amount of resin only binds 7 mg. The resin was used in prior assays of our study, which significantly increased their duration due to multiple centrifugation steps. This approach also required the availability of the LowBind tubes or silicone treatment of the tubes to minimize resin loss.

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Tissue homogenization and collection of control samples

For better preservation of plant tissue, leaf blades were wrapped in foil and placed in liquid nitrogen.

The precooled mortars and pestles were used for homogenization. The tissue was placed in a mortar, a small amount of liquid nitrogen was added, and the mixture was homogenized to a powder. The obtained powder was then added to a tube with 300 µl of lysis buffer composed of 50 mM Tris - HCl - buffer (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1 mM benzamidine HCl, 55 µM phenanthroline, 10 µM bestatin, 20 µM leupeptin, 5 µM pepstatin A, 1 mM PMSF, 1mM DTT, and 0.4 U/µl RiboLock™ (Thermo Fisher Scientific). The tubes were then sealed, mixed on a vortex mixer, and put on ice to cool down. The obtained homogenate was centrifuged at 4 ºC for 7 minutes at maximum speed. The supernatant was carefully transferred to clean precooled tubes without disturbing the debris. The obtained lysate had a rather high detergent content, which could potentially cause dissociation of RNA-protein complexes and weaken the protein's bond with the substrate. To avoid this, 700 µl of the buffer composed of 50 mM Tris - HCl (pH 7.4), 137 mM NaCl, 2.7 mM KCl was added to the lysate. At this stage, two control samples were collected: 1) lysate samples of 100 µl were placed in separate tubes for future analysis of RNA input fraction; 2) lysate samples of 10 µl were placed in 0.6 µl tubes to evaluate the binding efficacy between the target protein and the substrate. Both tubes were stored at 4 ºC.

Pull-down and wash of RNA-protein complexes

The tubes with the magnetic beads prepared earlier were placed on a magnetic stand, and the excess liquid was removed. The tubes were then removed from the stand, and the obtained diluted lysate was added to the beads. The tubes were placed on an orbital shaker, incubated at constant rotation for two hours at 4 ºC, and transferred back to the magnetic stand. Lysate samples of 10 µl were collected to control the protein's bond with the substrate, the remaining liquid was carefully removed.

The magnetic beads were washed by adding 400 µl of the buffer composed of 50 mM Tris - HCl (pH 7.4), 137 mM NaCl, 2.7 mM KCl, and 0.05% Igepal Ca-630 (Promega Corp.). The tubes were gently shaken manually three times and placed on the magnetic stand. It should be noted that the number of washes is chosen for each RNA-protein complex on an individual basis. The assay described here included five washes. The tubes were incubated on an orbital shaker for five minutes at 4 ºC during the last wash.

When the last wash was finished, the tubes were put on ice.

Evaluation of binding efficacy between protein and magnetic beads

At this stage, content, preservation, and binding efficacy of the target protein were evaluated. For this purpose, tubes with the previously collected lysate fractions described in sections "Tissue

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homogenization and collection of control samples" and "Pull-down and wash of RNA-protein complexes" were further analyzed. 1 µl of 50 µM HaloTag® TMR Ligand (Promega Corp.) was added to each tube. The content was mixed by pipetting, and the tubes were kept in the dark for 15 minutes. Then, 10 µl of 4x SDS loading buffer was added and the mixture was heated for 2 minutes at 90 ºC. We prepared 8% polyacrylamide gel for Laemmli electrophoresis [17] and placed 5 µl of the obtained product in gel wells. The gel was analyzed using a densitometric scanner Typhoon FLA 9000 (GE Healthcare) at the given wavelength (extinction wavelength of 532 nm and the emission wavelength of 580 nm).

It can be seen from Fig. 2 that a higher percentage of protein turned out to be bound and therefore not detectable in the supernatant after two-hour incubation. Otherwise, the go-to solution is to increase incubation time. A larger amount of magnetic beads increases non-specific binding.

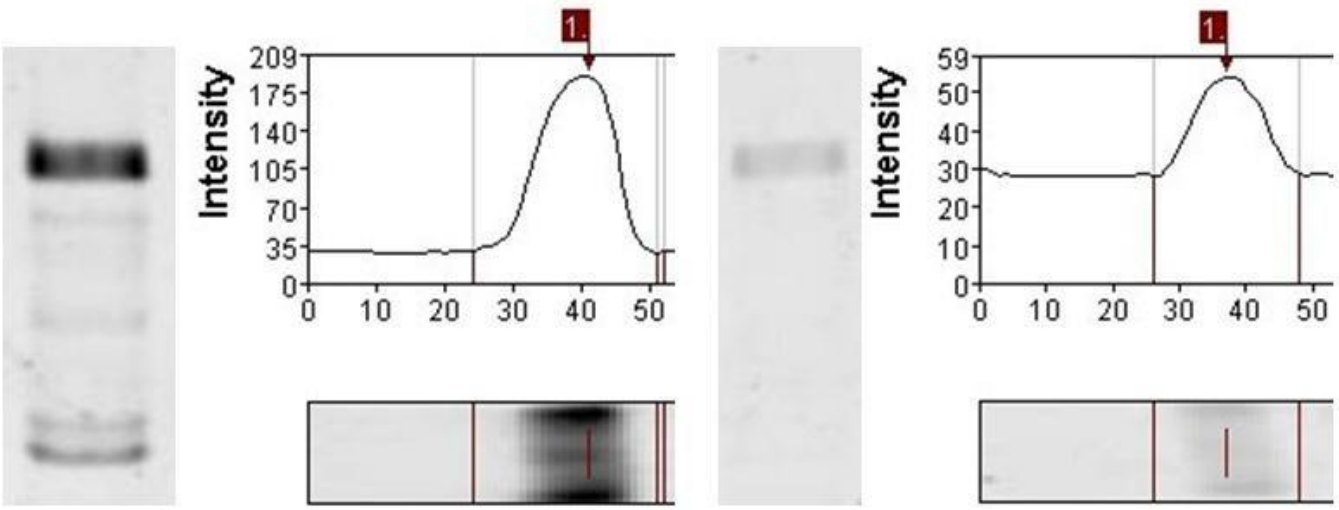

Fig. 2. Evaluation of the binding efficacy of the EsCSDP3 protein on magnetic beads using the TMRligand fluorescent dye. a) a lysate sample before binding and the fluorescence level of the bound dye; b) the lysate sample in 2 hours after incubation at 4 ºC and fluorescence level of the bound dye. The unbound protein fraction is about 25%.

RNA isolation

At this stage, two methods of isolating RNA from the protein complex are available, and prior knowledge of the studied protein, as well as the further course of analysis of the obtained RNA are to be taken into account.

For instance, isolating RNA directly from magnetic beads by incubation in the ExtractRNA reagent (Evrogen) (**first method**) produces the eluate, which, in addition to the target RNA obtained directly from the protein, includes several non-specifically bound RNA molecules from HaloTag® and the substrate. This isolation method is preferable, if the further analysis includes RT-

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PCR or Real-time PCR with primers on the known RNA targets.

If NGS is used to identify the nature of the unknown RNA targets, then elution in TEV buffer and further extraction using the ExtractRNA reagent (Evrogen) (**second method**) is recommended. TEV protease treatment of the RNA-protein complex facilitates its release into the solution, while non-specifically bound RNA molecules stay at the bottom of the tube.

To isolate RNA from the eluate, 100 µl of the buffer composed of 50 mM Tris - HCl (pH 8.0), 0.5 mM EDTA, 0.005 mM DTT, 40 U of RiboLock™ (Thermo Fisher Scientific) and 5 U of HaloTEV protease (Promega Corp.) was added to the washed beads with precipitated target RNA-protein complex. The tube was placed on an orbital mixer and incubated overnight at 4 ºC. The next day, the tubes were placed on a magnetic stand, and 90 µl of the eluate was transferred to a clean 1.5 ml tube. Then, 1 ml of ExtractRNA reagent (Evrogen) was added to the obtained eluate. At this stage, RNA isolation from the beads and from the eluate proceeded identically. We similarly added 1 ml of ExtractRNA reagent (Evrogen) to the tubes with magnetic beads washed in the buffer solution, and incubated the tubes on a magnetic stand at room temperature for five minutes with careful intermittent mixing. Then 200 µl of chloroform was added and the content was mixed on a vortex mixer for 30 seconds. The tubes were then centrifuged at 4 ºC at 10000g for 10 minutes. We

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carefully collected 500 µl of aqueous phase and transferred it to a new tube. We then added 25 µg of glycogen, mixed the content by pipetting, and incubated for 10 minutes at room temperature. The tubes were centrifuged for 10 minutes at 18000g at room temperature. The supernatant was carefully removed with a small amount of isopropanol left at the bottom, and 1 ml of 75% ethanol was added to the precipitate. The tubes were incubated at -20 ºC overnight. Then the tubes were centrifuged at maximum speed at room temperature for 5 minutes. The supernatant was carefully removed, and the precipitate was dried for 10 minutes at room temperature and eluted into 20 µl of RNase-free water.

Analysis of the obtained RNA

To measure the concentration of the obtained RNA, a Quantus Fluorometer (Promega Corp.) was used. RNA profile was analyzed using a 2100 Bioanalyzer with RNA 6000 Nano and Pico kits (Agilent). Due to the high sensitivity of the device, a sample volume of 1 µl was sufficient for analysis, and therefore enough eluate may be preserved for further experiments.

The RNA profile obtained using the 2100 Bioanalyzer is presented in Fig. 3.

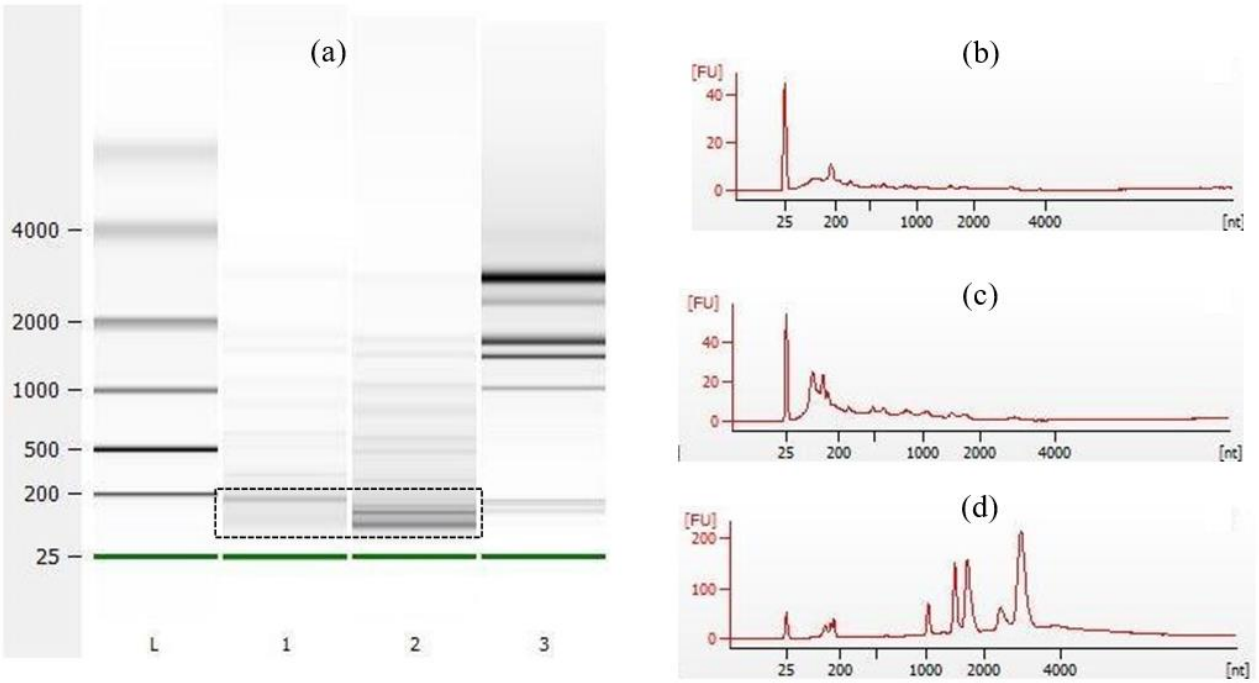

Fig. 3. RNA profile obtained using an Agilent 2100 Bioanalyzer with RNA 6000 Nano kit: (a-c) RNA is isolated using the first method, where a1 and b correspond to the HaloTag protein sample; a2 and c - to the sample of the EsCSDP3 protein; a3 and d - to the total RNA sample used for reference. A dashed outline indicates a small RNA zone. The RNA size compared to the reference marker is measured along the x-axis. The fluorescence intensity of the intercalating dye is measured along the y-axis.

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RNA obtained by elution from TEV buffer and further isolation using the ExtractRNA reagent (the second method) is shown in Fig. 3a. Comparison of the two RNA profiles shows that a much wider variety of various RNAs with higher RNA concentration was obtained by elution in the case of RNA-protein complex (plot 1), than in the case of HaloTag (plot 2). After more detailed consideration, we could notice that both samples had some common RNAs, and this should be taken into account in further analysis and comparisons. The small RNA zone in samples from Fig. 3a (dashed outline) is shown in Fig. 4.

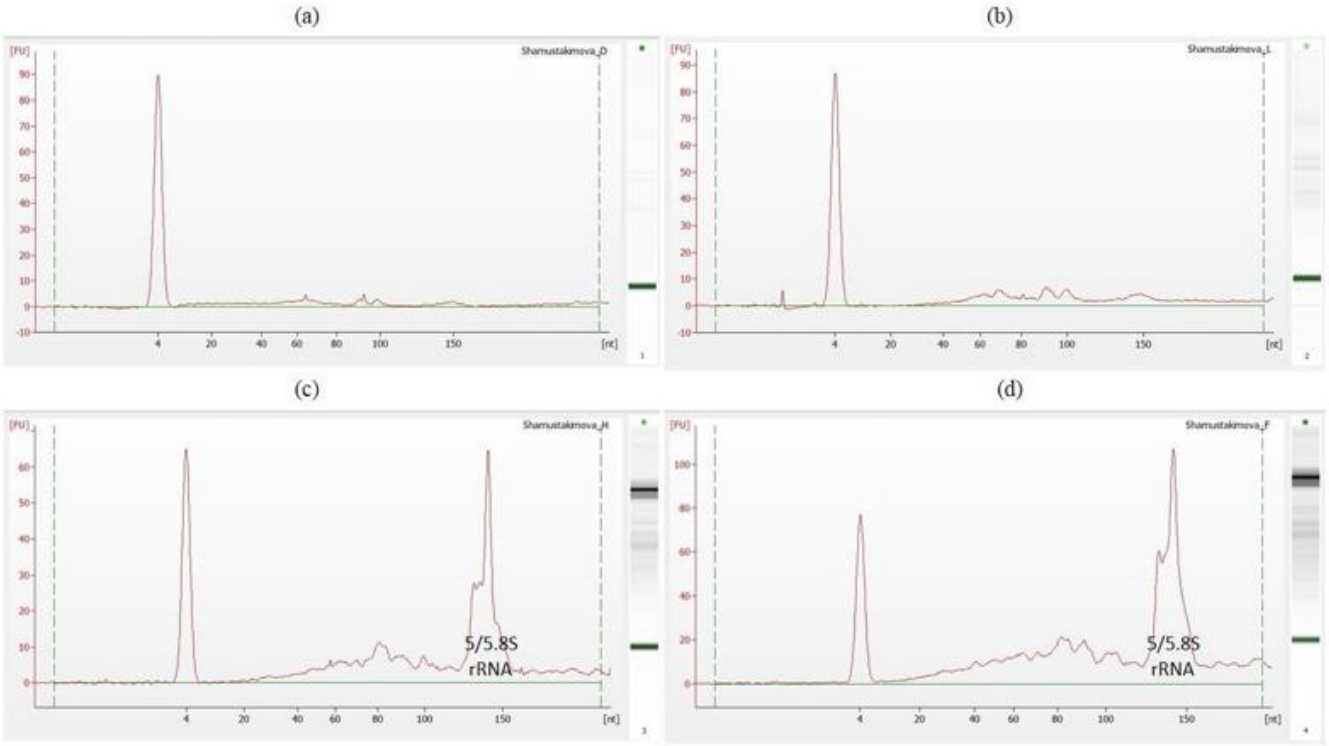

Fig. 4. RNA profile obtained using an Agilent 2100 Bioanalyzer with Small RNA kit for small RNA analysis: (a-c) a and b are the HaloTag and EsCSDP3 proteins samples from the assay showed in Fig. 3a (dashed outline); c and d show the RNA isolated from the HaloTag and EsCSDP3 proteins samples using the second method. The fluorescence intensity of the intercalating dye is measured along the y-axis.

This zone was of special interest, since major differences between the samples were found. Its analysis also made it possible to predict the nature of RNA targets. The small RNA zone may be divided as follows: miRNA at nucleotide counts of ~40 and below; transfer RNAs at nucleotide counts of ~40 to ~80; small ribosomal RNAs at nucleotide counts of ~80 to ~150.

As expected, comparison of the RNA profiles obtained using two different extraction methods showed a much higher fluorescence intensity in samples obtained using the first method (magnetic beads). In these samples, 5/5.8S ribosomal RNA made up the highest proportion of all RNA types.

Comparison of the total fluorescence of the HaloTag and EsCSDP3 samples showed that the signal of the experimental sample (110 FU) was almost twice as intense as that of the control sample (65 FU).

Preparation of cDNA libraries and sequencing

The proposed protocol does not include a detailed description of cDNA library preparation and further sequencing procedures. It only lists the aspects to be taken into account in assay designs.

A cDNA library preparation kit should be selected based on the amount of RNA obtained. Although the *A. thaliana* genome is relatively short, its number of genes is comparable to that of humans, specifically 27000 against 25000. Thus, the recommended read depth is 15-30 mln unpaired reads with lengths of 50 bp [18-19].

This read length is sufficient for mapping onto the genome, and the given read depth should be sufficient to identify specific targets based on their abundance quantitation in a statistically valid manner.

When RNA is obtained directly from magnetic beads, it is worth using a ribosomal RNA removal kit, since they would account for a large number of reads due to their high abundance.

The obtained results have shown that the proposed method makes it possible to isolate complexes of fusion proteins and RNA targets from the *A. thaliana* leaves.

The Halo-RPD method has a number of advantages compared to the protocols based on immunoprecipitation, in particular, stable protein expression makes it possible to minimize both the initial amount of plant material for the assay and reagent consumption. The use of the reagent at the stage of RNA isolation from the eluate/substrate allows one to obtain and analyze small RNA and miRNA. The absence of covalent crosslinks and removal of ultrasonic fragmentation stage make the proposed protocol applicable for analyzing native RNA profiles and thereby draw preliminary conclusions on the nature of RNA targets, while the use of fluorescent dyes covalently bound to HaloTag protein makes it possible to control the proper implementation of homogenization and protein isolation stages.

The author thanks Charles Banks from the Stowers Institute for Medical Research for assistance in protocol development. The work was supported by the Russian Foundation for Basic Research (project nos. 05-04-89005- NWO and 14-04-00816). The equipment was provided by the Common Use Center “Biotechnology” of the All-Russian Research Institute of Agricultural Biotechnology (project no. RFMEFI62114X0003).

The present paper does not include any studies with humans and animals as test subjects. The authors declare no conflict of interest.

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