Chapter 17

Tethered Function Assays to Elucidate the Role of RNA-Binding Proteins

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Abstract

The fate of each RNA molecule is strongly determined by RNA-binding proteins (RBPs) which accompany transcripts from its synthesis to its degradation. To elucidate the effect of a specific RBP on bound RNA, it can be artificially recruited to a specific site on a reporter mRNA that can be followed by a variety of methods. In this so-called tethering assay, the protein of interest (POI) is fused to the coat protein of the MS2 bacteriophage and expressed in your favorite cells together with a reporter gene containing MS2 binding sites. The MS2 binding sites are recognized by the MS2 coat protein (MS2CP) with high affinity and specificity and by doing so, the POI is tethered to the reporter RNA. Here, we describe how with the help of this assay the human cytoplasmic poly(A) binding protein is recruited to a mini-μ RNA reporter, thereby influencing the stability of the reporter transcript.

Key words RNA-binding protein, Tethering assay, Tethered protein, MS2 coat protein (MS2CP), MS2 binding sites, Poly(A) binding protein, Nonsense-mediated mRNA decay (NMD)

1 Introduction

RNA-binding proteins (RBPs) are essential for all steps in gene expression, ranging from synthesis and processing of transcripts in the nucleus to transport to the cytoplasm and localization within the cell. Furthermore, stability/turnover of transcripts and translation of mRNAs are heavily influenced and regulated by RBPs. Proteome-wide studies in different organisms showed that the number of RBPs was previously underestimated and it is now believed that more than 1500 RBPs are encoded in the human genome (reviewed in [1–3]). Equally important to the identification of these numerous RBPs is the careful elucidation of the function of each RBP, which may depend where on the mRNA it binds. Here, the tethered function assay makes a valuable contribution.

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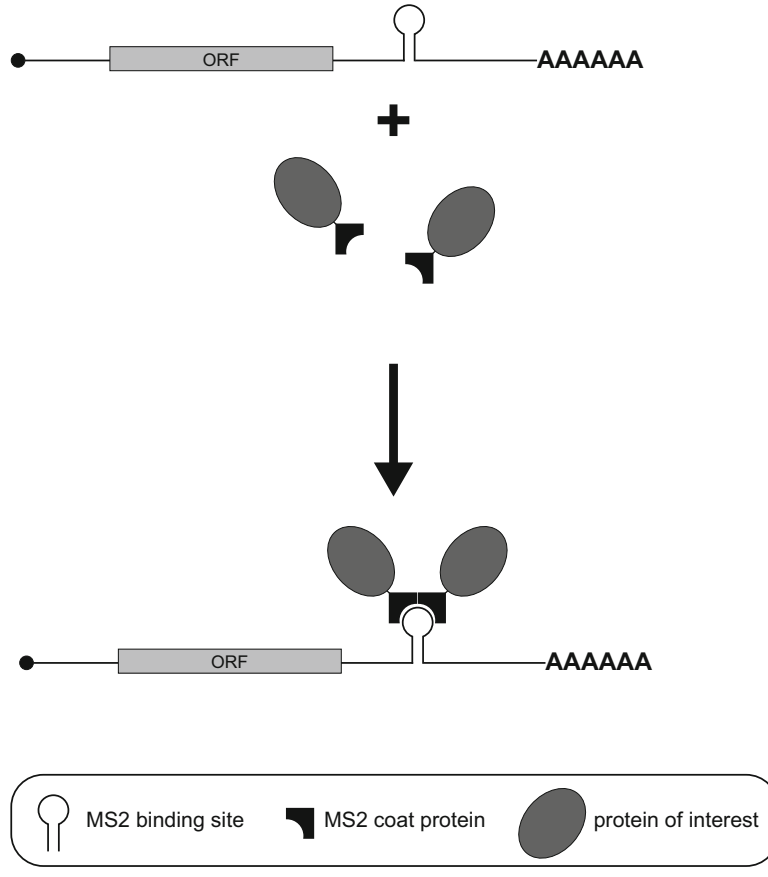

Fig. 1 A schematic overview of a tethered function assay where the protein of interest (POI) is tethered to the 3′UTR of a reporter mRNA via interaction of the MS2 coat protein (MS2CP) fused to the POI and the MS2 binding site located in the reporter transcript (for more details *see* introduction)

In a tethering assay experiment, an RBP is attached to a reporter RNA independently of its RNA-binding specificity and the impact of this RBP recruitment to the reporter RNA is examined. This allows to investigate proteins with unknown RNA substrates or even with no direct RNA binding capability (e.g., proteins indirectly associated with RNA molecule via protein-protein interaction). Two elements are needed in the tethering approach (Fig. 1): First, a specific RNA motif that serves as a high affinity binding site is introduced into a reporter gene (e.g., MS2 binding site in Fig. 1). The reporter gene is chosen in a way that the desired properties of the transcribed RNA can be easily followed (e.g., translation efficiency, RNA stability, processing or localization). Second, the protein of interest (POI) is expressed as fusion protein together with a moiety that binds the cognate binding site expressed on the reporter RNA (e.g., MS2 coat protein (MS2CP) in Fig. 1). If both parts are present in the cell, the POI is tethered

with high affinity and specificity to the reporter RNA and its effect on the reporter can be determined. The strength of a tethering experiment is that the impact of one protein is investigated in relation to one step in gene expression in a well-defined and controllable approach.

The focus of this chapter is on MS2 tethering, which was pioneered 1998 by Wickens's group [4] and used in numerous studies elucidating important functions of RBPs in RNA metabolism (for examples see Table 1). Similar tethering systems have been developed by using the λN peptide or the PP7 coat protein instead of the MS2CP to attach the POI to high affinity binding motifs in the reporter RNA, called BoxB binding sites for the λN peptide and PP7 hairpins for the PP7 coat protein. The PP7 tethering system is often used in combination with the MS2 system to investigate single mRNA molecules by microscopy [5, 6]. The λN peptide, the PP7 approach or other less commonly used tethering systems are not further discussed here, but concisely summarized in [7, 8].

In this chapter, the four steps of an MS2 tethering assay will be discussed to successfully investigate the effect of a POI on a specific readout of the reporter mRNA. Exemplarily, the protocol shows how the recruitment of the human cytoplasmic poly(A) binding protein (PABPC1) downstream of a stop codon in a reporter transcript leads to the stabilization of the mRNA molecule.

1.1 Designing and Cloning of Reporter Genes Containing MS2 Binding Sites

One of the most important considerations before starting a tethering experiment is choosing a suitable reporter gene, which strongly depends on the desired downstream application. For instance, to record translation efficiency Renilla luciferase is a good choice as luminescence detection is a fast and easy proxy for translation efficiency. In addition, it is also important that *cis*-acting features important for processing and stability of the reporter transcripts are characterized in detail. In our example, the abundance of a reporter mRNA with and without a tethered protein will be analyzed using reverse transcription followed by quantitative PCR (RT-qPCR) to characterize RNA stabilizing proteins. To this end, a version of an immunoglobulin μ (mini-μ) reporter gene harboring a premature translation termination codon (PTC) is used that has been studied extensively in the field of nonsense-mediated mRNA decay (NMD), a post-transcriptional mRNA surveillance pathway [9, 10] (Fig. 2a).

The localization of the MS2 binding sites in the reporter RNA is another critical aspect in the experiment design. Many examples show that the tethering position influences the outcome of the experiment and therefore testing different positions might be favorable. The RBP Staufen (STAU1) for instance elicits NMD when tethered to the 3′UTR, while attachment to the 5′UTR stimulates translation [11, 12]. It was even demonstrated that recruitment of proteins to the 5′UTR of reporter genes can lead to an inhibition of

Table 1
Short selection of MS2 tethering experiments

Analysis of		Protein of interest tethered	Comment	Reference
Abundance of mRNA	*Increased*	PABPC1 (poly(A) binding protein cytoplasmic 1)	PABPC1 tethering near a premature stop codon (3′ of PTC) suppresses NMD leading to increased reporter mRNA levels	[21]
	Reduced	UPF1, SMG6 (two proteins involved in NMD)	Tethering of NMD factors (e.g., UPF1 or SMG6) to 3′UTR of reporter mRNAs elicit NMD leading to the decay of the mRNA	[16, 15]
Translation efficiency	*Activation*	METTL3 (m6A methyl-transferase like 3)	METTL3 enhances translation when tethered close to a stop codon. Firefly luciferase is measured to determine translation efficiency	[22]
	Repression	TNRC6 (trinucleotide repeat containing 6; shown to interact with argonaute proteins)	Tethering of TNRC6b or TNRC6c to the 3′UTR of a luciferase RNA exerts a strong translation inhibition. Studies with mutant versions of TNRC6b indicate that ago-binding is not required for translation silencing	[23]
Alternative splicing		SRSF1	Tethering of SRSF1 to an identified *cis*-element in exon 4 in DOK7 pre-mRNA activates the intron-distal 5′ splice site and suppresses the intron-proximal 5′SS of DOK7 intron 4	[24]
RNA localization		GFP, YFP, mCherry	Tethering of fluorescent proteins is used to visualize localization of mRNAs in living cells	Numerous studies, discussed in [25]
	Transport	She3p	Tethering of She3p to lacZ reporter mRNA leads to the transport of the reporter mRNA to the bud tip in *S. cerevisiae*	[26]
	Site of transcription	–	Active site of transcription of a reporter gene was detected by RNA FISH with a Cy5-labeled probe that hybridizes to the MS binding sites of the reporter mRNA	[27]

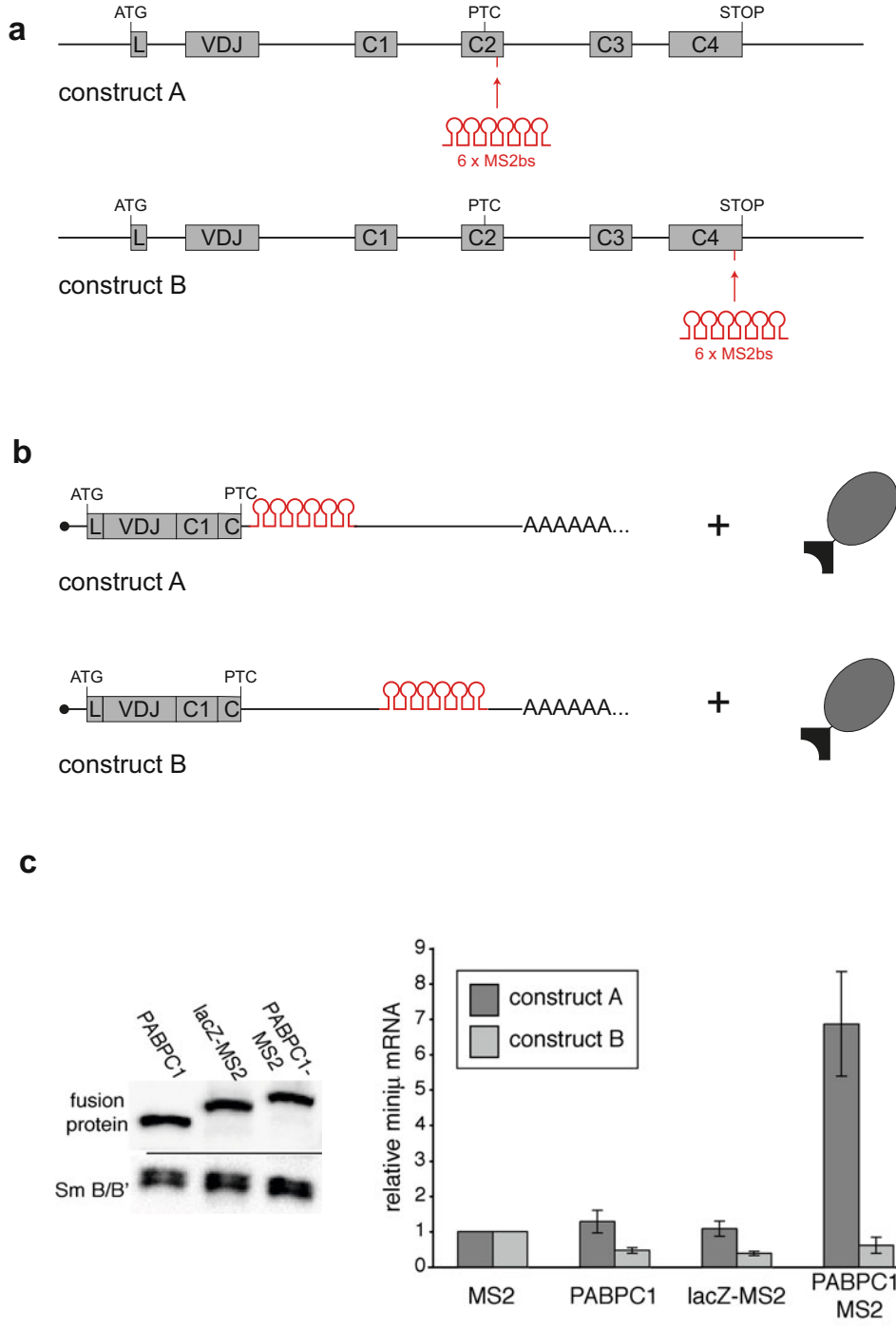

Fig. 2 Detailed workflow of the tethered function assays to elucidate the role of PABPC1 in mRNA abundance. (**a**) Insertion of six MS2 binding sites into a reporter plasmid (pβ mini-μ ter310) at two different positions (close to the PTC in construct A and further away in construct B). (**b**) Expression of these reporter plasmids in eukaryotic cells together with PABPC1 fused to MS2CP. (**c**) Left panel: Expression of PABPC1-MS2-HA fusion protein and the controls (PABBC1-HA without MS2CP or lacZ-MS2-HA) is analyzed by western blotting, SmB/B′ serves as loading control. Right panel: Relative mini-μ mRNA levels of construct A (dark bars) and construct B (light bars), normalized to cotransfected GPx1, is measured by RT-qPCR showing that tethering PABPC1 close to a termination codon leads to stabilization of the reporter mRNA (data taken from [21])

translation independent of the function of the protein [13]. The 3′UTR of reporter genes often represents a good starting choice for the addition of the binding sites, since this position does not prevent translation and is a natural binding platform for many RBPs. In the example outlined below, the MS2 binding sites were introduced at two different positions in the 3′UTR: either close to the premature stop codon or further away, eliciting different effects by the tethered proteins (Fig. 2a).

1.2 Cloning the MS2 Fusion Protein

The next step is to prepare a plasmid which contains the coding sequence of a fusion protein between the POI and the MS2CP, where the latter is crucial for tethering the protein to the reporter RNA. Generally, the MS2CP can be attached to the N- or C-terminus of the POI, but its expression has to be verified by western blotting. It can never be completely excluded that the fusion protein is folded improperly or that the MS2CP moiety disturbs the POI's function. A good strategy is thus to add the MS2CP to either terminus of the POI from the beginning. Furthermore, the addition of a GC linker helps to minimize the risk for sterical clashes between the POI and the MS2CP. The requirement of expression of a properly folded and functional fusion protein is an important limitation of the system. Only positive results are functionally meaningful while no effect on the reporter RNA could always be due to improperly folded and thus inactive proteins. In addition, several controls are necessary to monitor the quality of the experiment that are discussed in Subheading 3.2. If tethering of the POI affects the reporter, further insights into important domains or amino acid residues of the POI can be obtained by subsequently tethering mutant proteins to the reporter RNA and analyze their effect, for example, as shown for PABPC1 [14] or for the endonuclease SMG6 involved in degrading NMD sensitive transcripts [15]. Tethering the endonuclease to a reporter mRNA led to a reduced mRNA levels that is dependent on the catalytic active PIN domain of SMG6 since tethering mutant SMG6 (three crucial amino acids in the PIN domain are mutated) did not exhibited reduced RNA levels [15].

1.3 Expression of Both Constructs in Cells

The transfection of the reporter plasmid with the MS2 binding sites and the plasmid encoding the POI-MS2CP fusion protein to cells leads to the tethering of the POI to the reporter RNA by virtue of the MS2CP binding to the MS2 binding site. Expression of the reporter and effector constructs need to be carefully monitored to minimize indirect effects deriving from their overexpression in the cells.

Furthermore, elegant experiments can be conducted by combining knockdown experiments with the tethering approach, thereby investigating the interplay between two proteins. This approach was for example extensively and successfully used for

exploring the NMD pathway: The endonuclease SMG6 was tethered to a reporter RNA, leading to rapid degradation of this transcript as mentioned above. However, if the SMG6 tethering was combined with a knockdown of the NMD key factor UPF1, the level of the reporter RNA was not reduced and SMG6-mediated decay of the reporter mRNA impaired, demonstrating that the presence of UPF1 is required for SMG6 to exert its endonuclease activity [15].

1.4 Functional Analysis of the Protein of Interest

The functional study at the end of the experiment lies of course in the suspected function of the RBP and the reporter gene chosen in the beginning. Different downstream measurements can be performed to analyze the impact of the POI to RNA processing/splicing, stability, mRNA localization or translation (for examples *see* Table 1 and references therein): Processing of the reporter RNA can be assessed by northern blotting or analytical PCR, subcellular localization of RNA molecules can be observed by microscopy or by fractionation, and translation efficiency can be recorded by checking the protein abundance by luminescence (for luciferase), fluorescence (for GFP) or western blotting. In the example shown here, the levels of RNA molecules are determined by RT-qPCR addressing the question whether PABPC1 affects the abundances of transcripts.

2 Materials

2.1 Cloning of Plasmids

1. Oligonucleotides used for cloning are listed in Table 2.
2. DNA template for PCR.
   (a) to amplify MS2 binding sites, for example, β2-3MS2 containing six MS2 binding sites ([16] and *see* **Note 1**).
   (b) to amplify your POI, for example, pETNHis-hPABPC1 (obtained by Elmar Wahle, University of Halle, Germany).
3. Reporter plasmid where the MS2 binding sites will be introduced, for example, pβ mini-μ ter310 [9].
4. Expression plasmid encoding MS2CP, for example, pCMV-H2b-MS2-HA (*see* **Note 2**).
5. 10× buffer for high-fidelity polymerase (e.g., *pfu*Ultra high-fidelity reaction buffer, Agilent).
6. dNTP stock (10 mM each).
7. High-fidelity polymerase (e.g., *pfu*Ultra high-fidelity DNA polymerase, Agilent).
8. Tubes for PCR reaction and PCR machine.

Table 2
Oligonucleotides used for cloning and qPCR

Name	Sequence (5′–3′)	Notes
For cloning		
MS2 bs_for	CTAAGTTGTACAACCAAACTGGGTC TAGCTCTAG	To amplify MS2 binding sites (+*BsrGI* site)
MS2 bs_rev	ATTTAGTGTACACTATAGAATAGGGCCC TCTAG	To amplify MS2 binding sites (+*BsrGI* site)
PABPC1_for	CATGGTACCACCATGAACCCCAG TGCCCCCAGCTACCCCATGGCCTCGC TCTACGTG	To amplify PABPC1 (*KpnI* site)
PABPC1_rev	CATGGATCCGGAACAG TTGGAACACCGGTGG	To amplify PABPC1 (*BamHI* site)
For MS2-HA control_for	CACCATG	Annealed oligonucleotides for cloning of MS2-HA (*see* **Note 13**)
For MS2-HA control_rev	GATCCATGGTGGTAC	Annealed oligonucleotides for cloning of MS2-HA (*see* **Note 13**)
For PABPC1-HA control_for	GATCCATACCCATATGATGTTCCAGA TTACGCTTCACTCGAATGAGC	Annealed oligonucleotides for cloning of PABPC1-HA (*see* **Note 14**)
For PABPC1-HA control_rev	GGCCGCTCATTCGAGTGAAGCGTAATC TGGAACATCATATGGGTATG	Annealed oligonucleotides for cloning of PABPC1-HA (*see* **Note 14**)
lacZ_for	AAGGTACCACCATGGTGCGCTG TTCGCATTATCC	To amplify part of lacZ (*KpnI* site)
lacZ_rev	TTGGATCCGG TTTTTGACACCAGACCAACTG	To amplify part of lacZ (*BamHI* site)
For qPCR		
Mini μ assay:	Forward: GTCTCACCTTCTTGAAGAACGTGTC Reverse: GGGATGGTGAAGGTTAGGATGTC Taqman probe: FAM-CACATGTGCTGCCAGTCCCTCCAC -BHQ	To quantify mini μ mRNA: TaqMan probe lies over exon–exon junction (C2-C3)
GPx1 assay:	Forward: TGGTGGTGCTCGGTTTCC Reverse: GACATACTTGAGGGAA TTCAGAATCTC Taqman probe: FAM-CCATTCTCCTGATGTCCGAACTGA TTGC-TAMRA	To quantify GPx1 mRNA to normalize: TaqMan probe lies over exon–exon junction

9. Reagents and equipment for agarose gel: agarose, TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), ethidium bromide, loading buffer, DNA ladder.
10. Restriction enzyme of interest and corresponding buffer, for example, *Bsr*GI, *Bam*HI, *Not*I, *Kpn*I.
11. DNA purification kit (e.g., Wizard SV Gel and PCR Clean-Up kit, Promega).
12. Buffer for Antarctic phosphatase (50 mM Bis-Tris–Propane–HCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 6).
13. Antarctic phosphatase.
14. 10× buffer for T4 DNA ligase.
15. T4 DNA ligase.
16. 100 mM NaCl.
17. 10× buffer for T4 polynucleotide kinase (PNK).
18. 10 mM ATP.
19. T4 PNK.
20. Chemically competent *E. coli* XL1 blue.
21. Ampicillin (Amp) or Kanamycin (Kan).
22. Lysogeny broth (LB) medium.
23. LB agar plates.
24. Plasmid Miniprep kit.
25. NanoDrop instrument (UV spectrophotometer).

2.2 Expression of Constructs in Cells

1. Human cervix epithelioid carcinoma cell line (HeLa cells).
2. 37 °C incubator, 5% CO_2.
3. Dulbecco's modified Eagle's medium (DMEM).
4. DMEM +/+: DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin.
5. 6-well plates.
6. Transfection reagent (e.g., DreamFect, OZ Biosciences).
7. Plasmid to transfect for normalization, for example, pCMV-rGPx1 (*see* **Note 3**).
8. PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.
9. T/E: 0.05% trypsin–EDTA.
10. Neubauer counting chamber or cell counter.
11. SDS-PAGE loading buffer.
12. Lysis buffer for RNA analysis (from RNA isolation kit) or TRI-mix (*see* **Note 4**).

2.3 Functional Analysis of the Protein of Interest

1. RNase-free water: filtered or DEPC-treated.
2. RNA isolation kit (e.g., Absolutely RNA Miniprep Kit, Agilent) or reagent for TRI-mix extraction method.
3. Reagents for DNase treatment (e.g., TURBO DNA-free™ kit, Ambion).
4. Reagents for reverse transcription (RT): 150 ng/μL random hexamers, RT-buffer, 10 mM dNTP, RNase inhibitor, reverse transcriptase.
5. Reagents and equipment for qPCR: qPCR machine, qPCR tubes, 2× master mix for qPCR (e.g., Brilliant III Ultra-Fast qPCR Master Mix, Agilent).
6. Oligonucleotides and Taqman probes for qPCR are listed in Table 2.
7. General reagents and equipment for SDS-PAGE and western blotting.
8. Antibodies to detect tagged fusion proteins and a protein to normalize, e.g., anti-HA and anti-SmB/B′.
9. For analytical PCR: suitable oligonucleotides, Taq Polymerase, PCR buffer, dNTPs.

3 Methods

3.1 Designing and Cloning of Reporter Genes Containing MS2 Binding Sites

Before starting the experiment, decide which reporter gene is suitable for the desired readout and where the MS2 binding sites should be located in the reporter gene (see introduction for further discussion). Additionally, the number of the MS2 binding sites should be carefully evaluated. It was shown that two MS2 stem loops are sufficient for successful recruitment of a protein to the reporter gene [4, 16–18], but often more than two MS2 binding sites, up to 24 binding sites, are used, eventually increasing the detected effect of the RBP on the reporter RNA [19]. However, it has to be assessed that by adding a long sequence with strong secondary structure, processing or stability of the reporter transcript is not impaired. Additionally, 24 repeats of the MS2 binding site may cause problems to efficiently and correctly replicate such a plasmid in bacteria, often resulting in low plasmid yield and skipping of MS2 binding sites. If a cassette consisting of ≥12 binding site is needed, for example, to reach sufficient sensitivity for single molecule live cell imaging experiments [19] recombinase-deficient *E. coli* strains are recommended for plasmid amplification and for each plasmid preparation, the number of MS2 binding sites should be confirmed. Here, six MS2 binding sites were inserted ~70 nts downstream of a PTC (ter310) in the C2 exon of the pβ mini-μ ter310 plasmid (construct A, Fig. 2a) or further away, ~45 nts

upstream of the physiological termination codon in exon C4 (construct B, Fig. 2a). In particular, the MS2 binding sites were amplified by PCR and introduced into the *Bsr*GI restriction site located in the C2 exon (downstream of ter310) and in the C4 exon.

1. Find suitable restriction sites to introduce the MS2 binding sites into the reporter gene of choice and design oligonucleotides accordingly (*see* **Note 5**):

   Forward oligonucleotides:
   CTAAGTTGTACAACCAAACTGGGTCTAGCTCTAG-3'

   Reverse oligonucleotides:
   ATTTAGTGTACACTATAGAATAGGGCCCTCTAG-3'

   TGTACA: *Bsr*GI restriction site.

   bold: additional nucleotides for efficient digestion.

   underlined: complementary to template plasmid to amplify MS2 binding sites (sequences for MS2 binding sites *see* **Note 6**).

2. Perform PCR to amplify the six MS2 binding sites with *Bsr*GI overhangs at both sides. As template, pcβwt β2-3MS2 containing six MS2 binding sites is used [16]. Alternatively, any plasmid with MS2 binding sites can serve as template (for suitable plasmids from Addgene *see* **Note 1**). Pipette the following reagents in PCR tube: 1× PCR reaction buffer, 0.2 mM of each dNTP, 0.4 μM of each primer, 50 ng template DNA, and 2.5 U high-fidelity DNA polymerase.
3. Run the PCR according to the following scheme: 1 × (95 °C—2 min); 35 × (95 °C—30 s, 55 °C—30 s, 72 °C—30 s); 1 × (72 °C—5 min).
4. Analyze 5 μL of the PCR reaction on an 1.5% agarose gel (*see* **Note 7**).
5. Digest the PCR reaction with *Bsr*GI enzyme in the recommended buffer at 37 °C. Afterward, heat-inactivate the enzyme at 80 °C for 20 min.
6. Purify the amplified and digested PCR fragment by a PCR purification kit (*see* **Note 8**).
7. Digest reporter pβ mini-μ ter310 plasmid with *Bsr*GI. Since two *Bsr*GI sites are present in pβ mini-μ ter310 a partial digestion is necessary to get a linearized plasmid that is cut at only one *Bsr*GI site. For that prepare several restriction reactions and incubate each tube for a different time span at 37 °C (*see* **Note 9**).
8. Run an aliquot of each digestion reaction on an analytical agarose gel and decide for the best digestion condition to receive plasmids cut at one *Bsr*GI site. For comparison load undigested plasmid on the agarose gel.

9. Repeat the partial digestion with the decided condition and run the whole reaction on an agarose gel.
10. Purify the linear plasmid by cutting out the desired band from the agarose gel and follow the protocol of the purification kit.
11. Dephosphorylate linearized vector to reduce religation of the vector: Incubate purified plasmid with 5 U Antarctic phosphatase and corresponding buffer for 30 min at 37 °C. Heat-inactivate phosphatase at 80 °C for 2 min.
12. Insert the digested and purified PCR fragment either into the *Bsr*GI site in exon C2 (for construct A) or into *Bsr*GI site in exon C4 (for construct B) of pβ mini-μ ter 310 plasmid. For that, add the linearized and dephosphorylated pβ mini-μ ter 310 plasmid (from **step 11**) and the digested PCR fragment (from **step 6**) in a tube with T4 DNA ligase and the corresponding buffer (including ATP) in a molar ratio of 1:3 and incubate overnight at 16 °C (*see* **Note 10**).
13. Next day, transform the ligation reaction to chemically competent *E. coli* XL1 blue and plate them on LB plates containing ampicillin (Amp).
14. Analyze the colonies from the plate: Inoculate single colonies in 4 mL LB/Amp and cultivate them in a shaker (200 rpm) over night at 37 °C.
15. Isolate the plasmids by a Miniprep kit the next day.
16. Find correct plasmids by analysis restriction digestions on agarose gel. Here, the minipreps are digested with *Xba*I (cutting once in MS2 binding sites and once in mini-μ ter310 backbone) so that the orientation of the insert can also be determined.
17. Send plasmids for sequencing to confirm that they are correct.
18. The new constructs (construct A and construct B) have to be tested to confirm that the insertion of the MS2 binding sites does not disturb the stability and processing of the expressed RNA of the reporter plasmid. Splicing of the reporter RNA is analyzed by analytical RT-PCR as described in Subheading 3.4. To monitor the stability of the newly cloned plasmids, the RNA abundances of construct A and construct B can be measured by RT-qPCR and compared to the original plasmid. For that, transfect the original plasmid pβ mini-μ ter310t or pβ mini-μ ter310 construct A or pβ mini-μ ter310 construct B in HeLa cells and analyzed the RNA levels 48 hours post transfection by RT-qPCR (for details *see* Subheadings 3.3 and 3.4).

3.2 Cloning the MS2 Fusion Protein

Expression plasmids encoding a fusion protein between the POI and the MS2CP are needed as second piece for a functional tethering approach (Figs. 1 and 2c: PABPC1-MS2). The MS2CP of 14 kDa should be appended to the N- and C-terminus of the POI to test possible interference of the MS2CP moiety to protein's function (data not shown). In addition, several controls are needed to convincingly show that the effect detected relies on the POI's recruitment to the reporter RNA: (1) MS2CP alone (Fig. 2c: MS2), (2) POI without the MS2CP moiety (Fig. 2c: PABPC1), and (3) an unrelated protein of similar size as the POI fused with MS2CP (Fig. 2c: LacZ-MS2). Additionally, mutant versions of the POI are interesting to include as discussed in the introduction.

In all constructs, an HA-tag is added to monitor the expression of the proteins by western blotting. In this example, the MS2CP is fused to the C-terminus of the POI. However, attaching the MS2CP moiety to the N-terminus was also tested and no difference was found between the two positions of the MS2 protein.

3.2.1 Cloning of pCMV-PABPC1-MS2-HA

The starting plasmid for the generation of the MS2 fusion plasmids was a plasmid containing the open reading frame of histone H2b fused to the sequence encoding the MS2CP and an HA-tag (pCMV-H2b-MS2-HA). H2b is flanked by *Kpn*I and *Bam*HI and can therefore be easily replaced with any sequence of a POI that is amplified by PCR with flanking *Kpn*I and *Bam*HI sites (*see* **Note 11** for sequence information). Any other plasmid containing the sequence of the MS2CP can be used as a starting point for cloning or as template for PCR amplification (*see* **Note 2**).

1. Design forward and reverse oligos to amplify the open reading frame of your POI and include the desired restriction sites at the end. Here, the primers used to amplify the open reading frame of PABPC1 contain a *Kpn*I (forward oligonucleotide) and *Bam*HI (reverse oligonucleotide) restriction site and a few additional nucleotides at the end to enable efficient binding and cutting of the respective restriction enzymes (oligonucleotides are listed in Table 2).
2. Run PCR to amplify the open reading frame of the POI with *Kpn*I and *Bam*HI overhangs at the sides. As template pETNHis-hPABPC1 is used (obtained by Elmar Wahle, University of Halle, Germany). For that, pipette 1× PCR reaction buffer, 0.2 mM of each dNTP, 0.4 μM of each primer, 50 ng template DNA and 2.5 U high-fidelity DNA polymerase to a PCR tube and run the reaction in a PCR cycler according to the T_m of the oligonucleotides, length of the amplicon, and properties of the polymerase.
3. Digest the amplicon with *Kpn*I and *Bam*HI at 37 °C.
4. Run the digested amplicon on an agarose gel and purify it via a purification kit (*see* **Note 8**).

5. Digest the vector pCMV-H2b-MS2-HA with *Kpn*I and *Bam*HI at 37 °C to cut out the H2b insert of about 400 bp.
6. Add phosphatase and corresponding buffer to the restriction reaction to dephosphorylate the vector. Incubate the reaction for 30 min at 37 °C.
7. Run the digested and dephosphorylated reaction on an 1% agarose gel. Cut out vector backbone band and purify via a purification kit (*see* **Note 12**).
8. Ligate vector backbone with PCR amplified and digested PABPC1 (molar ratio 1:3) over night at 16 °C to generate pCMV-PABPC1-MS2-HA (as done before in Subheading 3.1, **step 12**).
9. Transform the ligation reaction to competent *E. coli* XL1 blue and plate them on LB plates containing kanamycin (Kan).
10. Analyze the colonies by restriction analysis and sequencing of the isolated plasmids (as described above in Subheading 3.1, **step 14**).

3.2.2 Cloning of Controls: MS2CP Alone (pCMV-MS2-HA)

1. Use the digested and purified vector backbone from **step 7** (H2b sequence removed).
2. Design two oligonucleotides that can anneal and close the digested vector backbone: 5′-CACCATG-3′ and 5-′-GATCCATGGTGGTAC-3′. The Kozak sequence is included to increase translation initiation (*see* **Note 13** for clarification).
3. Anneal the two oligonucleotides: Mix 10 μL of each oligonucleotides (10 μM each) and add 20 μL 100 mM NaCl (final concentration: 50 mM). Incubate the tube at 95 °C for 5 min, slowly cool to room temperature.
4. Phosphorylate the annealed oligos with 10 U T4 polynucleotide kinase (PNK) in 1 × PNK reaction buffer and 1 mM ATP at 37 °C for 30 min. Heat-inactivate the enzyme by incubating at 65 °C for 20 min.
5. Ligate the annealed and phosphorylated oligonucleotides with the digested vector backbone and continue as described above for cloning of pCMV-PABPC1-MS2-HA (**steps 9** and **10**).

3.2.3 Cloning of Controls: PABPC1 Without MS2CP (pCMV-PABPC1-HA)

The cloning strategy of pCMV-PABPC1-HA is the same as for pCMV-MS2-HA. pCMV-PABPC1-MS2-HA is digested with *Bam*HI and *Not*I to remove MS2-HA. The complementary oligonucleotides (5′-GATCCATACCCATATGATGTTCCAGATTAC GCTTCACTCGAATGAGC-3′ and 5′-GGCCGCTCATTCGA GTGAAGCGTAATCTGGAACATCATATGGGTATG-3′) are annealed, phosphorylated, and ligated into the vector backbone (cut by *Bam*HI and *Not*I) bringing back the HA-tag, but not the MS2 sequence (*see* **Note 14**).

3.2.4 Cloning of Controls: Unrelated Protein with MS2CP (pCMV-lacZ-MS2-HA)

Follow the protocol described for cloning of pCMV-PABPC1-MS2-HA, but design primers to amplify a sequence encoding a control protein with no function in RNA metabolism. In our example here, a C-terminal fragment of lacZ (1910 bp) of similar size than PABPC1 was amplified by PCR, including suitable restriction sites at both ends (*Kpn*I and *Bam*HI).

3.3 Expression of Constructs in Cells

After confirming the cloned plasmids by sequencing, they are transfected to eukaryotic cells leading to POI's tethering to the MS2 binding sites of the reporter RNA (Fig. 2b). Here, we use HeLa cells cultivated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/mL streptomycin.

1. Seed 2×10^5 HeLa cells in 6-well plates and incubate overnight at 37 °C, 5% CO_2.
2. Transfect the plasmids the next day. Titration experiments for finding optimal plasmid concentrations have been performed before (*see* **Note 15**). Set up transfection reaction accordingly.
3. Prepare the following four plasmid mixtures for **mini-µ construct A** in 100 µL DMEM −/−: 100 ng mini-µ construct A (6 MS2 binding sites in C2 exon, *see* Subheading 3.1); 100 ng pCMV-rGPx1 (for normalization, *see* **Note 3**); 300 ng MS2-HA or PABPC1-HA or lacZ-MS2-HA or PABPC1-MS2-HA (*see* Subheading 3.2).
4. Prepare the following 4 plasmid mixtures for **mini-µ construct B** in 100 µL DMEM −/−: 100 ng mini-µ construct B (6 MS2 binding sites in C4 exon, *see* Subheading 3.1); 100 ng pCMV-rGPx1(for normalization, *see* **Note 3**); 300 ng MS2-HA or PABPC1-HA or lacZ-MS2-HA or PABPC1-MS2-HA (*see* Subheading 3.2).
5. Prepare the transfection reagent mixture in a tube: 4 µL transfection reagent DreamFect and 96 µL DMEM −/− per transfection (*see* **Note 16**).
6. Mix 100 µL of the transfection reagent mixture with 100 µL plasmid mixture and incubate for 20 min at room temperature.
7. In between wash cells once with PBS and add 1 mL DMEM −/−.
8. After 20 min, add each 200 µL DreamFect-plasmid mix dropwise to the cells in the 6-well plate.
9. Change medium 3–4 h later and add DMEM +/+.
10. Change the medium once again on the following day.
11. Harvest the cells 48–52 post transfection: Wash cells once with PBS, add a few drops T/E, and incubate for 5–10 min at 37 °C.

Resuspend cells in DMEM +/+, put them on ice, and count them by cell counter or with the help of a Neubauer counting chamber.

12. For Western Blotting, add 2×10^5 cells to a tube, centrifuge for 5 min at 200 rcf (4 °C) and remove supernatant. The cells are lysed in SDS-PAGE loading buffer and stored at −20 °C until usage (continue in Subheading 3.4, Western Blot).
13. For RNA analysis, spin down the residual cells (5 min, 200 rcf, 4 °C), remove supernatant and resuspend pellet in 1 mL TRI-mix or Lysis Buffer of an RNA Isolation kit (*see* **Note 4**).

3.4 Functional Analysis of the Protein of Interest

Finally, the effect of the tethered protein to the reporter transcript can be analyzed. Here, the **abundance of the reporter RNA** in the different conditions is measured **by RT-qPCR**:

1. Isolate total RNA by a suitable kit (e.g., Absolutely RNA Miniprep kit) or by TRI-mix (*see* **Note 4**).
2. Degrade residual DNA by DNase treatment (e.g., Turbo DNA-free kit or add DNase to the column when using RNA isolation kit).
3. Measure RNA concentration by nanodrop.
4. Reverse transcribe 1 μg RNA to cDNA. To control possible DNA contamination, include RT control samples in which the reaction is performed as depicted below, but substitute the reverse transcriptase with H_2O. A specific RT protocol follows: add 1 μg RNA, H_2O and 300 ng random hexamer in a tube (total volume 41 μL). Incubate for 5 min at 65 °C to remove secondary structures. Cool down at room temperature for 10 min. Add RT buffer, 0.4 mM dNTPs, 40 U RNase inhibitor, 50 U reverse transcriptase to the tube (total volume 50 μL). Incubate for 60 min at 42 °C. For inactivation of the RT incubate at 85 °C for 10 min. Add 75 μL DEPC-treated H_2O to the tube (→8 ng/μL).
5. Run a quantitative PCR (qPCR, real-time PCR) with 40 ng of reverse transcribed RNA per reaction using assays to quantify mini-μ mRNA and GPx1 mRNA levels (TaqMan probe [200 nM], forward and reverse oligo [800 nM each]). Include in the same run H_2O as nontemplate control and RT control samples to detect any DNA contamination.
6. Analyze the effect of the tethered POI in the RT-qPCR as in Fig. 2c.

Two important controls have to be conducted to convincingly show the effect of the tethered protein. First, the **expression of the fusion proteins** in HeLa cells has to be detected **by western blotting**:

7. Run whole cell lysates corresponding to 2×10^5 HeLa cells (*see* Subheading 3.3, **step 12**) per lane on a 10% SDS-PAGE.
8. Transfer proteins to a nitrocellulose membrane.
9. After blocking the membrane with 5% milk powder dissolved in TBS–Tween for 30–60 min, add the first antibody (α-HA and α-SmB/B′ antibody diluted in 5% milk–TBS–tween) to the membrane and incubate overnight in the cold room.
10. Wash the membrane three times for 5 min with TBS–Tween and incubate membrane with corresponding secondary antibody for 1 h at room temperature.
11. Wash the membrane with TBS–Tween and detect proteins with standard method of the laboratory. SmB/B′ serves as loading control (*see* Fig. 2c for results and **Note 17**).

Second, the **correct processing of the reporter transcripts** expressed HeLa cells has to be examined **by analytical RT-PCR**:

12. Use the cDNA prepared in **steps 1–4** or prepare cDNA using oligo(dT) as primer (*see* **Note 18**).
13. Amplify reverse transcribed material corresponding to 200 ng by PCR using Taq polymerase, 0.4 μM of forward and reverse oligonucleotides in 1× PCR buffer (including dNTPs). For checking splicing of the reporter RNA, use primers flanking the insertion site of the MS2 binding sites. For construct A, primers complementary to a sequence in C1 exon (forward) and C4 exon (reverse) are used; for construct B, primers anneal to C2 exon (forward) and the 3′ UTR (after C4 exon, reverse).
14. Analyze end-point RT-PCR amplicons on a 1.25% agarose gel to confirm that splicing is not impaired.

4 Notes

1. The following Addgene plasmids can serve as templates to amplify MS2 binding sites:
   (a) pCI-TPI_WT-xrRNA-4MS2-4H (#108370, Niels Gehring).
   (b) pSL-MS2-6X (#27118, Robert Singer).
   (c) pSL-MS2-12X (#27119, Robert Singer).
2. The following Addgene plasmids contain the sequence of the MS2CP:
   (a) MS2_GFP (#61764, Daniel Larson).
   (b) phage UBC NLS-HA-2XMCP-tagRFPt (#64541, Jeffrey Chao).

3. An unrelated normalizer plasmid, which codes for an mRNA that is not present in HeLa cells, is used to compensate for varying transfection efficiencies in different samples.
4. RNA can be isolated by column-based methods such as the silica-based purification or by Guanidium Thiocyanate-Phenol-Chloroform extraction. For the latter extraction method, a TRI-mix consisting of water-saturated phenol, chloroform and a chaotropic denaturing agent called guanidinium thiocyanate is necessary (e.g., TRIreagent (Sigma-Aldrich), TRIzol (Invitrogen)). The protocol for producing a "homemade" TRImix can be found in [20].
5. Newer cloning strategies that are independent on restriction sites (e.g., In-Fusion® Cloning (Takara), Gibson Assembly® (New England Biolabs), GeneArt Seamless Cloning (Invitrogen)) as well as gene synthesis technology are of course applicable for all the cloning procedures.
6. Sequence of amplicon containing the six MS2 binding sites, flanked by *Bsr*GI sites:

Tgtacaaccaaactgggtctagctctagctgtagaaaacatgaggat**c**acccatgtctgctggacgactgt

agaaaacatgaggat**c**acccatgtctgctgtctagctgtagaaaacatgaggat**c**acccatgtctgctgga

cgactgtagaaaacatgaggat**c**acccatgtctgctgtctagctgtagaaaacatgaggat**c**acccatgtc

tgctggacgactgtagaaaacatgaggat**c**acccatgtctgctgtctagagggccctattctatag**tGTAC**

A

Underlined: complementary to oligonucleotides used in PCR.

21 nt RNA stem loops are marked in gray; each stem loop contains a mutation (**c in bold** instead of U in original stem loop) leading to a higher affinity for MS2CP [7].

7. A single DNA band of 297 bp should be present on the agarose gel if the indicated primers and template is used.
8. Purify the digested PCR amplicon directly by a PCR purification kit if a single band is detected on the agarose gel. If more than one band is detected, run the digested PCR product on a new preparative agarose gel, cut out the expected band and extract the amplicon from the agarose gel piece according to the protocol of the purification kit.
9. Partial digestions can be performed in different ways and have to be empirically determined for each restriction enzyme. It is recommended to change only one parameter, either time or amount of enzyme, per experiment. If the enzyme concentration is changed, use for instance a 1:10 dilution so that in the end reaction 20 U, 2 U, 0.2 U, 0.02 U, and 0.002 U of the

enzyme is used and incubated at 37 °C for 20 min. Stop the reaction immediately by addition of EDTA (10 mM final concentration) or by heating it up to 80 °C for 20 min. Otherwise keep the amount of enzyme constant and prepare five restriction reactions that are incubated at 37 °C but stop the reaction at different time points, e.g., after 5, 10, 15, 30, 60 min. Another common strategy that can be combined with the conditions above is the addition of ethidium bromide to the reaction to inhibit efficient second cutting of the restriction enzyme.

10. To control the preparation of the vector backbone (digestion and dephosphorylation), it is recommended to perform a non-insert control. To do so, add the same amount of vector to the ligation buffer and the ligase, but substitute the insert with H_2O.
11. A part of the sequence of the pCMV-H2b-MS2-HA plasmid depicting the three restriction sites ***Kpn*I**, ***Bam*HI**, and ***Not*I**:

ggtaccgccaccatgctatgccagagccagcgaagtctgctcccgccccgaaaaagggctccaagaag

gcggtgactaaggcgcagaagaaaggcggcaagaagcgcaagcgcagccgcaaggagagctattccat

ctatgtgtacaaggttctgaagcaggtccaccctgacaccggcatttcgtccaaggccatgggcatca

tgaattcgtttgtgaacgacattttcgagcgcatcgcaggtgaggcttcccgcctggcgcattacaac

aagcgctcgaccatcacctccagggagatccagacggccgtgcgcctgctgctgcctggggagttggc

caagcacgccgtgtccgagggtactaaggccatcaccaagtacaccagcgctaa**gGATCC**AATGGCTT

CTAACTTTACTCAGTTCGTTCTCGTCGACAATGGCGGAACTGGCGACGTGACTGTCGCCCCAAGCAAC

TTCGCTAACGGGATCGCTGAATGGATCAGCTCTAACTCGCGTTCACAGGCTTACAAAGTAACCTGTAG

CGTTCGTCAGAGCTCTGCGCAGAATCGCAAATACACCATCAAAGTCGAGGTGCCTAAAGGCGCCTGGC

GTTCGTACTTAAATATGGAACTAACCATTCCAATTTTCGCCACGAATTCCGACTGCGAGCTTATTGTT

AAGGCAATGCAAGGTCTCCTAAAAGATGGAAACCCGATTCCCTCAGCAATCGCAGCAAACTCCGGCAT

CTACGCG**TACCCATATGATGTTCCAGATTACGCTTCACTCGAATGA**GCGGCCGC

Underlined: H2b sequence.

Bold, gray: MS2CP sequence.

Bold, black: HA-tag sequence.

12. If the indicated plasmid and primers are used, the expected bands on an agarose gel are 4,382 bp for vector backbone (desired) and 390 bp for the H2b insert.

13. The oligonucleotides contain part of the Kozak consensus sequence (GCCRCC**ATG**G; start codon in bold) and by annealing the two oligonucleotides, the necessary sticky ends of the restriction enzymes are generated (***Kpn*I** and ***Bam*HI** site):

5´-**C**ACCAT**G**-3´

3´-**CATGG**TGGTA**CCTAG**-5´

14. The annealed oligonucleotides have sticky ***Bam*HI** and ***Not*I** ends. In addition, the sequence of the **HA-tag (bold)** is introduced in the oligos:

5´-**GATCC**A**TACCCATATGATGTTCCAGATTACGCTTCACTCGAATGA**GC-3´

3´-

GT**ATGGGTATACTACAAGGTCTAATGCGAAGTGAGCTTACT**CGCCGG-5´

15. Before starting the real experiments, it is strongly recommended to identify the optimal amount of the reporter plasmid (containing the MS2 binding sites) for transfection (suggested range: 50–500 ng/well in 6-well plates). If a condition with reasonable readout is found (assessable by, e.g., C_t value in RT-qPCR), the plasmid expressing the MS2 fusion protein should be also titrated (suggested range 300–1500 ng/well in 6-well plates).
16. To minimize differences in transfection efficiencies, a master mix for the transfection reagent mixture should be prepared. For 8 transfection reactions, mix 34 µL DreamFect (4 µL × 8.5) with 816 µL DMEM −/− (96 µL × 8.5) as master mix.
17. MS2CP has a molecular weight of 14 kDa and is not shown on the western blot. The calculated molecular weight of PABPC1-HA, LacZ-MS2-HA, and PABPC1-MS2-HA are 71 kDa, 87 kDa, and 85 kDa, respectively.
18. To enrich the synthesis of cDNA deriving from mRNA, the RT reaction can be performed with oligo(dT) as primer instead of random hexamers (use 200 nM 5′-d$(T)_{30}$VN-3′).

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