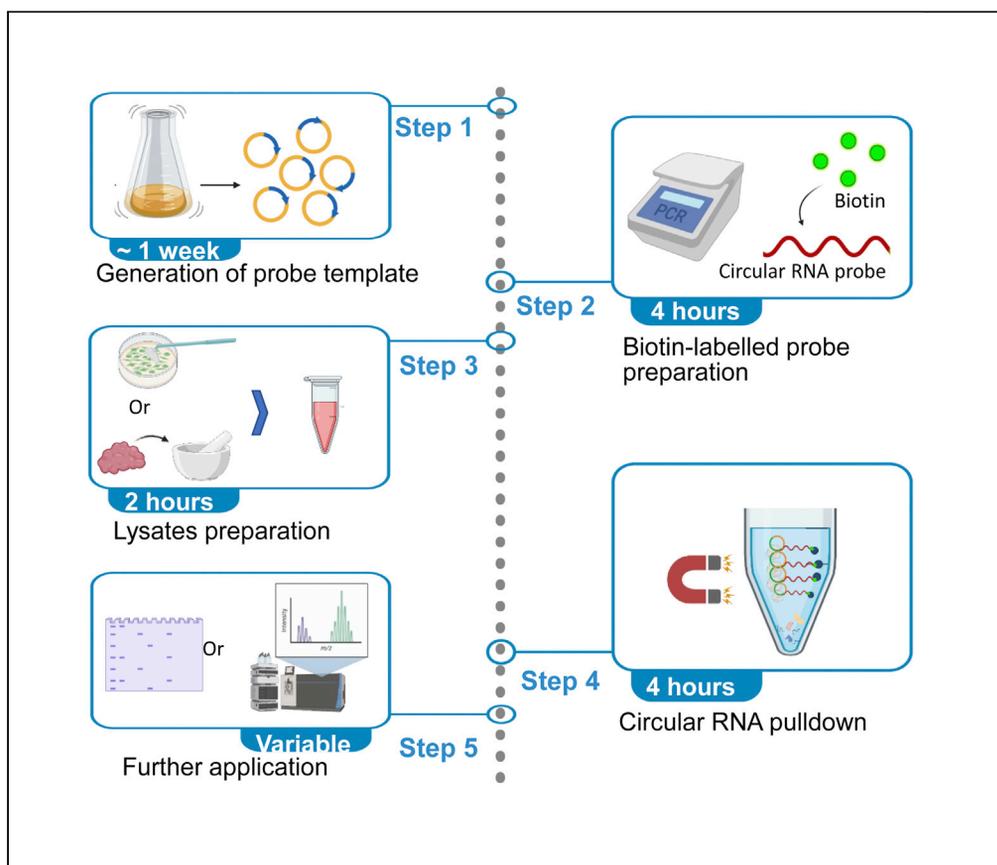


## Protocol

# Design and use of a back splicing junction probe for pulldown of circular RNA-binding proteins in cell lines



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**Highlights**  
Pulldown of circRNAs  
and their binding  
partners from MCF  
cells

Design of back  
splicing junction-  
based probe to  
achieve specificity to  
circRNA

The probe contains  
four repeats allowing  
efficient pulldown of  
the circRNA

Compatible with  
downstream assays,  
including RNAseq  
and proteomics

Due to the unique structure of circular RNAs, it is challenging to use traditional pulldown approaches. Here, we describe the design and use of a probe that spans the back splicing junction (BSJ), enabling interaction with circular RNAs. The probe repeats four times, allowing efficient and specific pulldown of circular RNAs and their binding partners. This protocol describes the steps for mouse cardiac fibroblast (MCF) cells; we have also verified the protocol in other cell types.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Protocol

## Design and use of a back splicing junction probe for pulldown of circular RNA-binding proteins in cell lines

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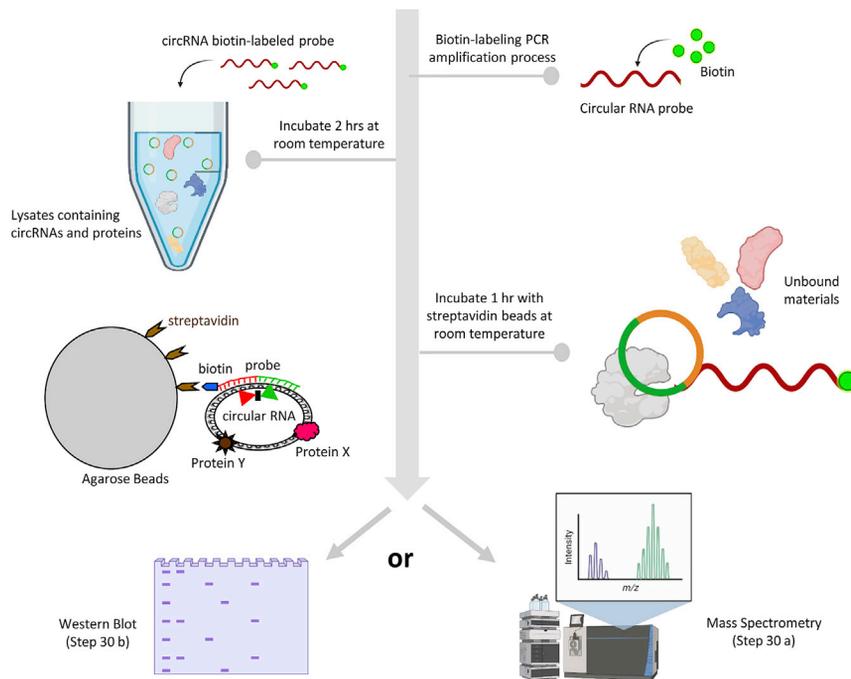
## SUMMARY

Due to the unique structure of circular RNAs, it is challenging to use traditional pulldown approaches. Here, we describe the design and use of a probe that spans the back splicing junction (BSJ), enabling interaction with circular RNAs. The probe repeats four times, allowing efficient and specific pulldown of circular RNAs and their binding partners. This protocol describes the steps for mouse cardiac fibroblast (MCF) cells; we have also verified the protocol in other cell types. For complete details on the use and execution of this protocol, please refer to Wu et al. (2021).

## BEFORE YOU BEGIN

Here, we present a standardized protocol to pull down circRNAs and their binding partners (Figure 1). The protocol below describes the specific steps for studying circRNA-protein interactions using mouse cardiac fibroblast (MCF) cells. However, the protocol is also verified and optimized through experiments with different aims using different cell lines (Fang et al., 2018; Wu et al., 2019, 2021). The protocol starts with the synthesis of a probe with four repeats that specifically recognizes the Back Splicing Junction (BSJ) of a circRNA of interest. There was a small fragment of 8-nt between each repeat to avoid spatial obstacle between the probe and the target circRNA. At both ends of the probe, two fragments, each with 18-nt, were added to allow specific PCR amplification of the probe. In the presence of biotinylated CTP, the primers amplify the probe sequence incorporating the biotinylated CTP into the probe. Incubation of the synthesized probe with lysates allows only the circRNA of interest to be recognized and tagged but no other non-circularized sequences (i.e., the mRNA from the same gene), which reduces the rate of false positive results. Following circRNA tagging by the biotinylated probe, the labeled circRNA is pulled down using streptavidin-coated beads. Any potential binding partners recognized by the circRNA will be thereby pulled down as well. Further applications following this pull-down procedure are various based on the need. There are notable differences between the protocol used by others in the field and the one we are presenting: i) we cloned the probe with 4 repeats that specifically recognizes the BSJ (the joint sequence: 18 nucleotides upstream and 18 nucleotides downstream) under T7 promoter of the plasmid to maximally increase the binding specificity and efficiency between the probe and the circRNA; ii) we optimized the amount of biotin CTP in the probe synthesis to ensure the best tagging process. In our design, the biotin CTP could be changed to any other biotin nucleobase depending on which nucleobase presents the most in the probe sequence to achieve optimal labeling efficiency; iii) the ratio between the forward primer and the reverse primer in the biotin-probe synthesis process was adjusted to 1:4 to allow the formation of large quantity of single-stranded probe for better pull-down results; iv) we introduced a pre-treatment of probe step before the pull-down, which involves a denaturation step at 95°C and immediately followed by incubation in icing water. This



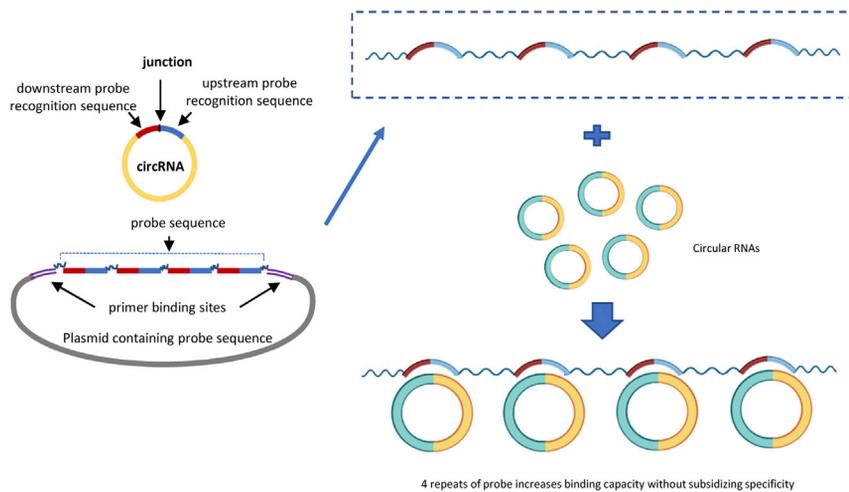


**Figure 1. General workflow of the circular RNA pull-down assay**

The workflow includes probe template generation, amplification, and biotin-labeling process. Lysates containing target circular RNA and its binding proteins should be prepared and incubated with biotin-labeled probe for 2 h at 25°C. The mixture will be further incubated with pre-washed streptavidin beads for 1 h at 25°C. A couple of intense washing steps will be performed following the finishing of the incubation periods and unbound materials should be washed off at this point. Further applications following the pull-down assay will be based on the research needs.

step allows the probe to dominantly stay in a single-stranded form rather forming a double-stranded structure by annealing, further ensuring the binding capacity between the probe and the circRNA; v) a number of wash steps were included according to different application purposes to remove as much non-specific binding as possible. This revised protocol increases binding specificity/sensitivity and allows the detection of any potential binding partners of the target circular RNA, i.e., this method is not limited to identifying direct circRNA-protein interactions, but it could also be used by other researchers who study circRNA-miRNA, circRNA-mRNA, circRNA-miRNA-mRNA axis, or circRNA-miRNA-protein axis regulatory mechanisms. Researchers in the circRNA-RNA field can simply adapt to this pull-down method followed by the use of commercialized RNA extraction kits. In summary, this approach can be used to identify the interaction of an endogenous (or recombinant) molecule (no matter it is an RNA or a protein) with the *in vitro*-synthesized probe against the circRNA of interest.

This protocol does not necessarily require a transfection step. However, it is recommended to over-express the circular RNA plasmid along with the vector plasmid control to achieve better comparison for later procedures especially if the target circular RNA has an endogenous expression level lower than 0.2 copies per cell (0.2–0.5 copies per cell would be good). Also, a higher endogenous expression with 1 copy per cell should have no problem with endogenous pull-down procedure. In addition, cells transfected with different plasmids (for example, vector control, circRNA, linear counterpart of circRNA, linear mRNA) or siRNAs (endogenous knockdown of circRNA) and followed by pull-down assay could further validate the information on the interactions between the circRNA and its binding partners.



**Figure 2. Diagram showing the principle of circular RNA probe design**

Probe sequence is designed to specifically recognize the BSJ sequence of the target circular RNA. Four repeats of the junction sequence are designed and cloned into a plasmid vector. A short space sequence of 8 nucleotides was added between the BSJ sequence allowing each repeat of the probe to bind to a circRNA. This would ensure the maximum binding affinity to the circular RNA without sacrificing the specificity of the binding site recognition. Primers used to amplify the probe are designed to complement to the sequences added to each probe. In our studies, 5'CAGCTCAGAGTGCTTTG and 5'GATGACTTTCCCGGCC were added to the 5' and 3' of each probe.

### Generation of a probe template

⌚ Timing: ~ 1 week, 10 h hands on

1. Design circRNA probes to specifically recognize the BSJ of the circRNA of interest (Figure 2). See Table 1 for examples.
2. Design and clone four repeats of probe sequences of the circRNA of interest into a plasmid.
3. Add a short space sequence of 8 nucleotides between the junction sequence allowing each repeat of the probe to bind to a circRNA without spatial obstacle.
4. Add sequences: 5'CAGCTCAGAGTGCTTTG and 5'GATGACTTTCCCGGCC to the 5' and 3' of each probe allowing PCR amplification of the probe.
5. Design and clone a control oligo probe with random sequence into another plasmid of the same kind. This oligo probe sequence should be at a size similar to the target circRNA probe.

⚠ **CRITICAL:** 4 repeats of the probe sequence ensure the binding efficiency of the probe to the circRNA of interest.

6. The designed sequence can be synthesized by a biotech company, so that it is convenient to order a plasmid-based template. Any plasmid backbone can be used. The one used in our studies was the Bluescript backbone.
7. Dissolve the plasmids according to the manufacturer's guidelines (Plasmid mini prep) using ddH<sub>2</sub>O.
8. Amplify the plasmids in bacteria and extract the plasmids to be used as probe templates with the Plasmid Extraction Kit (Figures 3 and S1). Miniprep is the most common choice for probe template making (Choose MaxiPrep, MediPrep or MiniPrep based on the volume required). Skip this step if adequate templates have been ordered from the company.
9. Determine the concentration and the quality of the plasmids using NanoDrop spectrophotometer.
10. Store the plasmids containing the probe templates at –20°C or –80°C for longer storage.

**Table 1. Examples of designed probe for pulldown**

Name	Probe sequence <sup>a</sup>	Primers
Probe A (circYAP)	5' CAGCTCAGAGTGTCTTTG gaactgcttcggcaggctccttctctga tg <b>acgttgca</b> gaactgcttcggcaggctccttctctgatg <b>aaccgggtt</b> gaact gcttcggcaggctccttctctgatg <b>ccggttga</b> gaactgcttcggcaggctcctc ttctctgatg <b>taaccgg</b> GATGACTTTCCCGGGCCC 3'	5' cagctcagagtgtctttg 5' gggcccgggaaagtcac
Probe B (circITG $\alpha$ 9)	5' CAGCTCAGAGTGTCTTTG gtggcccatggaagagcaaggcctgt cat <b>acgttgca</b> gtggcccatggaagagcaaggcctgtcat <b>aaccgggtt</b> gt ggcccatggaagagcaaggcctgtcat <b>ccggttga</b> gtggcccatggaagag caaggcctgtcat <b>taaccgg</b> GATGACTTTCCCGGGCCC 3'	5' cagctcagagtgtctttg 5' gggcccgggaaagtcac
Probe C (circNlg)	5' CAGCTCAGAGTGTCTTTG tcccgactgaggatggatagacctgcag <b>acgttgca</b> tcccgactgaggatggatagacctgcag <b>aaccgggtt</b> tcccgactgag gatggatagacctgcag <b>ccggttga</b> tcccgactgaggatggatagacctgc ag <b>taaccgg</b> GATGACTTTCCCGGGCCC 3'	5' cagctcagagtgtctttg 5' gggcccgggaaagtcac

<sup>a</sup>In each probe sequence, there are four repeats containing 30 nucleotides targeting the junction sequences of the circRNAs. These 30 nucleotides were obtained from the 5' end and the 3' end of the circRNAs. A short space sequence (8 nucleotides, bolded) was added between the repeats. Two extra sequences were added at the 5' and 3' of the four repeats (Upper cap) to facilitate probe amplification.

## Design and prepare primers

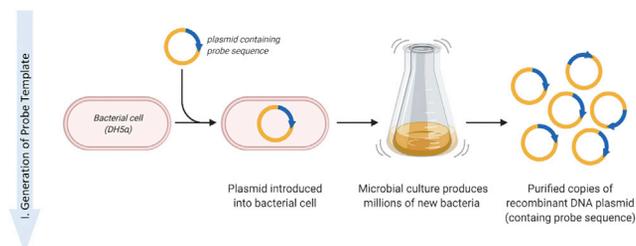
⌚ Timing: variable

- The forward and reverse primers with a length of 18 nt and melting temperature of  $\sim 55^{\circ}\text{C}$  are designed using Primer 3 online tool (<http://bioinfo.ut.ee/primer3-0.4.0/>). See Table 1 for examples. Primers used to amplify the probe are designed to complement to the sequences added to each probe.
- Order primers based on the need from a biotechnology company.
- Dissolve the primers in nuclease-free water to a stocking concentration of 100  $\mu\text{M}$ , then prepare final concentration of 10  $\mu\text{M}$  for each primer in nuclease-free water (e.g., add 10  $\mu\text{L}$  of each primer from the 100  $\mu\text{M}$  stock into 980  $\mu\text{L}$  of nuclease-free water and mix well).

## Overexpression of circular RNA plasmids in MCF cells

⌚ Timing: variable

Overexpression of a circular RNA is not a necessary step in all the pull-down experimental designs. For easier illustration and collective presentation of the expected results following this protocol, we mentioned an overexpression condition in the specific case of studying circYAP binding partners



**Figure 3. Workflow for Part I**

Generation of Probe Template (Timing  $\sim 1$  week, 10 h hands on). Probe template is designed and ordered from a biotechnology company. Through transformation, plasmid containing the probe sequence is introduced into the bacterial cells DH5 $\alpha$ . Following the bacterial cells culturing with kanamycin (or choose other antibiotics according to plasmid generation instruction), shake at  $37^{\circ}\text{C}$  overnight or at least 16 h, recombinant DNA plasmid containing the probe sequence will be purified using plasmid extraction kits (See Figure S1 for detailed information for miniprep plasmid extraction).

(prepared for mass spectrometry analysis). For further detailed information about transfection using PolyJet, please refer to “PolyJet *In Vitro* DNA Transfection Reagent — A General Protocol for Transfecting Mammalian Cell” and follow the manufacturer’s instructions. Alternatively, lipofectamine transfection reagents could be used, especially for hard-to-transfect cells.

14. Plate MCF cells (6 × 100 mm culture dishes per group) 18–24 h prior to transfection so that the monolayer cell density reaches to the optimal 70%–80% confluency at the time of transfection. Assign groups based on the experimental design. Here we present two groups: (i) vector control group, and (ii) circYAP group.
15. On the day of transfection, discard culture medium and use PBS wash once.
16. Add 5 mL serum-free medium to each dish.
17. Add polyjet at 3 μL/μg plasmid into 100 μL serum-free medium in a 1.5 mL Eppendorf tube.

©CAUTION: Never use Opti-MEM to dilute PolyJet™ reagent and DNA, it contains serum and will disrupt the transfection complex.

18. Add 5 μg/dish plasmid into 100 μL serum-free medium in a 1.5 mL Eppendorf tube.
19. Add the diluted PolyJet™ reagent immediately to the diluted DNA solution all at once.

©CAUTION: Do not mix the solutions in the reverse order.

20. Immediately pipette up and down 3–4 times or vortex briefly to mix.
21. Incubate for 10–15 min at 25°C to allow PolyJet™/DNA complexes to form.

©CAUTION: Never keep the PolyJet™/DNA complex longer than 20 min.

22. Add the PolyJet™/DNA mixture drop-wise onto the medium in each dish and homogenize the mixture by gently swirling the plate.
23. Remove PolyJet™/DNA complex-containing medium and replace with fresh complete serum/antibiotics containing medium 12–18 h post transfection.

**Note:** For sensitive cells, to lower cytotoxicity, remove PolyJet™/DNA complex and replace with complete medium 5 h after transfection.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Critical commercial assays</b>		
Taq DNA Polymerase Kit (5 U/μL) contains 10× PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), Magnesium Chloride (50 mM)	Thermo Fisher Scientific, Invitrogen™	cat.no. 10342-020
Presto™ Mini Plasmid Kit	FroggaBio, Geneaid™	cat. no. PDH300
<b>Experimental models: Cell lines</b>		
Mouse cardiac fibroblast (MCF) cells	This paper	This paper
<b>Other</b>		
dNTP Set (100 mM)	Thermo Fisher Scientific, Invitrogen™	cat.no. 10297018
Biotin-16-dCTP (1 mM)	Jena Bioscience	cat.no. NU-809-BIO16-S
Dynabeads™ MyOne™ Streptavidin C1 (10 mg/mL)	Thermo Fisher Scientific, Invitrogen™	cat.no. 65002
Protease Inhibitor Cocktail Set I	Millipore, Calbiochem	cat. no. 539131
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Thermo Fisher Scientific	cat. no. 10777019
LB Broth	WISENT Inc	cat.no. 800-060-LG

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Kanamycin Monosulfate	WISENT Inc	cat.no. 400-145-IG
AGAROSE D1-LE	WISENT Inc	cat.no. 800-015-CG
10× Tris/glycine/SDS running buffer	Bio-Rad	cat.no. 1610732
Nuclease-free water	Thermo Fisher Scientific, Ambion™	cat.no. AM9930
Bio-Rad protein assay dye reagent concentrate	Bio-Rad	cat.no. 500-0006
EDTA disodium salt (50 mM)	Calbiochem	CAS 6381-92-6
Ethidium Bromide	Sigma-Aldrich	E1510
NP-40 Surfact-Amps™ Detergent Solution	Thermo Scientific™	Cat.no. 85124
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich	D5758
PCR machine	Eppendorf Mastercycler Personal	N/A
Benchtop minicentrifuge with PCR strip tube rotor	Thomas Scientific	cat.no. C1201-E
Rotated shaker	N/A	N/A
16-Tube SureBeads™ Magnetic Rack	Bio-Rad	cat.no.1614916
NanoDrop One spectrophotometer	Thermo Fisher Scientific, Thermo Scientific	cat.no. ND-ONE-W
SmartSpec Plus spectrophotometer or other spectrophotometer with 595 nm wavelength	Bio-Rad Laboratories	cat.no.1702525
Refrigerated (4°C) centrifuge	Eppendorf	model: 5430R
Ultraviolet transilluminator	N/A	N/A
Vortexer	N/A	N/A
Magnetic stirrer	N/A	N/A
Cell scrapers	N/A	N/A
Homogenizer	N/A	N/A
100 mm cell culture dishes	N/A	N/A
1.5 mL Eppendorf tubes	N/A	N/A
Pipettes and filter tips (2.5 µL, 10 µL, 20 µL, 200 µL and 1,000 µL)	N/A	N/A
1.5 mL Disposable cuvettes	N/A	N/A

**Note:** Dynabeads™ MyOne™ Streptavidin C1 (10 mg/mL) should not be substituted for alternates. Other reagents/equipment could be substituted for similar versions from other companies. Specific alternate recommendations could be found in main text.

## MATERIALS AND EQUIPMENT

### Reagents setup

- dNTP set.

Prepare dNTP set according to the company's instructions. Cat. no. 10297-018 includes 100 mM each of dATP, dCTP, dGTP, and dTTP. Each vial contains 250 µL 25 µmol dNTP in purified water (pH 7.5). Mix 250 µL dATP, 250 µL dGTP, 250 µL dTTP, 125 µL dCTP and 125 µL purified water to make dNTP set. Aliquot the prepared dNTP set to 100 µL per 1.5 mL Eppendorf tube. Store at -20°C until further use.

dNTPs	Volume
dATP	250 µL
dGTP	250 µL
dTTP	250 µL
dCTP	125 µL
Purified water pH 7.5 (provided in the set)	125 µL
Total	1 mL

- Prepare buffers for pull-down procedure.

co-IP buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 7.5 (200 mM)	20 mM	10 mL
NaCl (1.5 M)	150 mM	10 mL
EDTA pH 8.0 (10 mM)	1 mM	1 mL
100% NP40	0.5%	0.5 mL
DEPC H <sub>2</sub> O	N/A	89.5 mL
<b>Total</b>	<b>N/A</b>	<b>100 mL</b>

Prepare following buffer amounts based on the requirement. Please refer to Dynabeads manufactory instructions (Dynabeads manufactory) for further details.

Buffer A	
Reagent	Final concentration
DEPC-treated NaOH	0.1 M
DEPC-treated NaCl	0.05 M

©CAUTION: Dissolve NaOH powder with DEPC water. Handle with extra care for autoclaving.

Buffer B	
Reagent	Final concentration
DEPC-treated NaCl	0.1 M

Stocking Washing buffer (2× Washing buffer)	
Reagent	Final concentration
Tris-HCl (pH 7.5)	10 mM
EDTA (pH 8.0)	1 mM
NaCl	2 M

Prepare ready-to-use 1× Washing buffer: Dilute 2× Washing Buffer with an equal volume of DEPC water. Add 0.01% [v/v] Tween™ 20.

**Note:** Buffers in this section could be prepared and stored at 4°C for up to 6 months.

- Other buffers.

50× stocking TAE buffer (1 L)	
	Amount
Tris-base	242 g
100% glacial acid (or acetic acid)	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL
	Top up to 1 L with ddH <sub>2</sub> O

Store stock solution at 25°C.

**Note:** No need for further adjustment of pH. Dissolve 242 g of Tris-base in 700 mL of ddH<sub>2</sub>O, mix well using magnetic stirrer before adding other reagents.

Prepare ready-to-use 1× TAE buffer: Dilute 20 mL 50× TAE buffer with 980 mL ddH<sub>2</sub>O, make it to 1 L 1× TAE buffer. Final concentration of each reagent in 1× TAE buffer is listed as follow:

1 × TAE buffer (1 L)	Amount
Tris-base	40 mM
100% glacial acid (or acetic acid)	20 mM
0.5 M EDTA (pH 8.0)	100 mM

DEPC water	Amount
0.1% Diethylpyrocarbonate (DEPC)	1 mL
	Top up to 1 L with ddH <sub>2</sub> O

Mix well using magnetic stirrer and let it set at 25°C for at least 1 h. Autoclave.

## STEP-BY-STEP METHOD DETAILS

### Biotin-labeled probe generation

⌚ Timing: ~ 4 h

In this section, circular RNA probes are labeled through biotinylation using PCR. Confirmation of successful labeled probe will be ensured in this section.

1. Prepare the PCR reaction as follows in 200 μL PCR tubes (Principle behind each setup is shown in [Figure 4](#)):

Reagents	CircRNA probe (μL)	Positive control (μL)	Negative control (μL)
Buffer	2	2	2
MgCl <sub>2</sub>	0.5	0.5	0.5
Biotin-16-dCTP	1	–	1
dNTP set	1	1	1
Forward primer	0.5	0.5	0.5
Reverse primer	2	2	2
Taq polymerase	0.1	0.1	0.1
Plasmid (template)	1	1	–
ddH <sub>2</sub> O	11.9	12.9	12.9
Total	20	20	20

⚠ **CRITICAL:** It is important to make sure that the reverse primer is added at least at a 4:1 ratio to the forward primer to ensure the formation of a desired circ-probe product. **DO NOT** use 1:1 primer ratio in this reaction.

⚠ **CRITICAL:** The Biotin-16-dCTP could be changed to Biotin-16-dGTP or Biotin-16-dATP or Biotin-16-dTTP. This depends on which nucleobase presents the most in the probe sequence.

**Note:** We provided in the table as volume for easier preparation. The recommended minimal concentration of template plasmid should be 200 ng/μL.

2. Mix the reaction mixture by tapping the tube for 5 s and centrifuge the PCR tubes for 5 s using minicentrifuge to settle the reaction mixtures at the bottom of the tubes.
3. Set up the Mastercycler program as follows:

1	T=94.0°C	0:02:00
2	T=94.0°C	0:00:30
3	T=56.0°C	0:00:30
4	T=72.0°C	0:01:00
5	GO TO STEP 2	Repeat for 30 cycles
6	T=72.0°C	0:05:00
7		HOLD at 4°C
		End

- Resolve the PCR products on an ethidium bromide stained 2% agarose gel for 30 min at 100 volts and visualize with an ultraviolet transilluminator.

**Note:** Positive control fragment should show a strong single band at the expected size (188 bp); Target fragment should show a clear single band at the expected size (Figure 4, slightly larger than positive control owing to biotin label); Negative control fragment should show blank. (? [troubleshooting](#)).

- Optimize the best conditions for probe generation and prepare the probes in larger volume according to the experimental design.

**Note:** A volume of 200  $\mu\text{L}$  is suggested for most conditions.

- Prepare both the oligo probe and the circular RNA probe as follows:

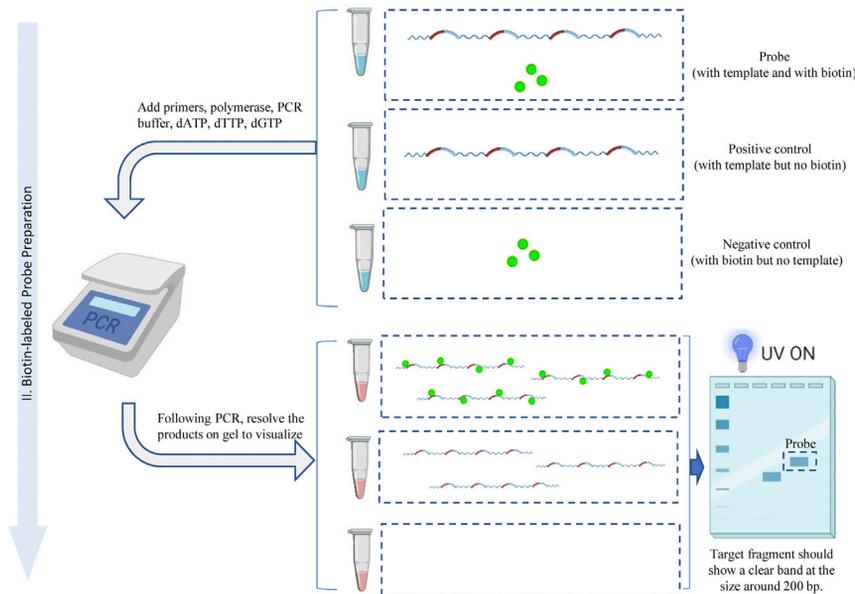
Reagents	Probe
Buffer	20 $\mu\text{L}$
MgCl <sub>2</sub>	6 $\mu\text{L}$
Biotin-16-dCTP	15 $\mu\text{L}$
dNTP set	10 $\mu\text{L}$
Forward primer	2 $\mu\text{L}$
Reverse primer	8 $\mu\text{L}$
Taq polymerase	2 $\mu\text{L}$
Plasmid (template)	1 $\mu\text{g}$
ddH <sub>2</sub> O	add up to 200 $\mu\text{L}$
Total	200 $\mu\text{L}$

**△ CRITICAL:** It is important to make sure that the reverse primer is added at least at a 4:1 ratio to the forward primer to ensure the formation of a desired circ-probe product. **DO NOT** use 1:1 primer ratio in this reaction).

©CAUTION: 50  $\mu\text{L}$  is usually the maximum volume for a thermal cycler to react. Make sure to aliquot the prepared 200  $\mu\text{L}$  PCR mix into 50  $\mu\text{L}$  per tube.

- Set up the Mastercycler conditions as step 3, or an alternative condition based on the pre-test results.
- Store the biotin-labeled probes at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  for longer storage, or use the probes immediately for the pull-down assay.

**▮▮▮ Pause point:** The probe can be stored at  $-20^{\circ}\text{C}$  for 3 months or  $-80^{\circ}\text{C}$  for longer periods.



**Figure 4. Workflow for Part II**

Biotin-labeled Probe Preparation (Timing ~ 4 h). A set of probe samples with small volumes (20  $\mu$ L) should be prepared for pre-test condition to test out the optimal conditions for probe generation. This should include a probe sample, a positive control sample, and a negative control sample. While the probe sample contains all the required ingredients (including primers, polymerase, PCR buffer, dATP, dTTP, dGTP and biotin CTP), the positive control contains everything except for biotin CTP, and negative control contains everything except for plasmid DNA. Following PCR procedure, probe sample should be biotin-labeled while controls should not. Resolving the PCR products on gel to visualize should obtain a clear band slightly bigger in bp size comparing with the positive control, where negative control should show blank.

### Reaction lysate preparation

⌚ Timing: ~2 h

In this section, you will prepare samples which you would like to explore binding partners of your target circular RNA.

As the circular RNA and protein yield varies between the cell lines, users should adjust the number of cells needed to extract at least 500  $\mu$ g proteins for each pull-down assay. For users who would like to subject the pull-down products for Mass Spectrometry as further application, at least 10 mg proteins for each pull-down assay should be prepared to ensure that at least 1 mg proteins should be pulled down and sent for mass spectrometry analysis.

**⚠ CRITICAL:** It is important to make sure that the protein concentration meets the requirements for further application. Please consult with Mass Spectrometry Centre for minimum concentration requirement for detection. This might be different for different machines.

**Note:** The co-IP buffer is prepared in DEPC water. All experimental consumables including tubes and tips should be all RNase-free. We did not add additional RNase inhibitors. However, if the further application focus on detecting following RNA binding partners, adding RNase inhibitors is necessary in addition to proteinase inhibitors. See following specific steps for Note.

9. Prepare cell or tissue samples following a. Cell lysates or b. Tissue lysates.

- a. Cell lysates.
  - i. Discard the culture medium with suction system in 10-cm dishes of ~90% confluent cells of interest. Wash three times with PBS and collect them using a cell scraper.
  - ii. Pellet the cells in a 1.5 mL tube by centrifuging at 25°C for 5 min at 500 ×g. If dealing with the cells with poor adhesive ability, collect the cells using the cell scraper first, pellet the cells as described in this step, disrupt and wash the cells with PBS. Repeat this cycle for 3 times.
  - iii. Fully discard PBS. Add 400 μL per tube of ice-cold co-IP buffer (1% proteinase inhibitor added).

**Note:** Add RNase inhibitors according to manufacturer's guidelines RNase inhibitor in addition to proteinase inhibitor.

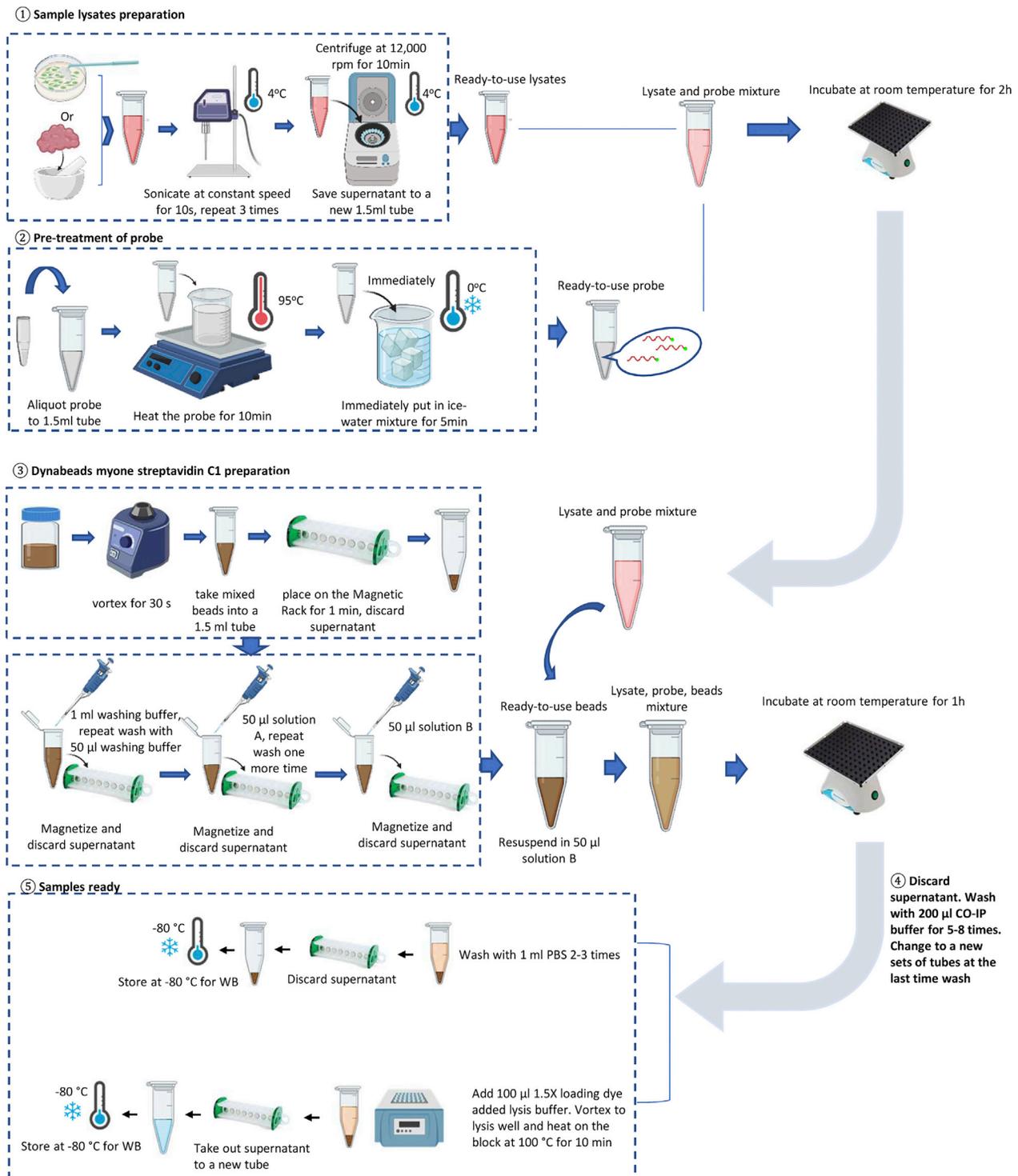
- iv. Pipette the samples up and down for 10 times and ensure that the pellets are fully disrupted.
- b. Tissue lysates.
  - i. Homogenize the tissue samples in 700 μL ice-cold co-IP buffer (1% proteinase inhibitor added) until no obvious tissue chunks are seen. This step needs to be performed on ice. An ice bucket is optimal. A homogenizer is recommended in this process. If using handheld mechanical homogenizer, please follow these cleaning up steps to avoid cross contamination:
  - ii. Prepare two sets of 15 mL tubes. The number of tubes in each set should equal to the sample size.
  - iii. Prepare 7 mL 3% H<sub>2</sub>O<sub>2</sub> (30% H<sub>2</sub>O<sub>2</sub> dilute in DEPC water) in each tube in one set of 15 mL tubes. Prepare 7 mL DEPC water in each tube in the other set. Set all the tubes on ice.
  - iv. Put the tip of the homogenizer in 3% H<sub>2</sub>O<sub>2</sub> tube, switch to "ON", hold for 5 s, and switch to "OFF". Repeat twice.
  - v. Put the tip of the homogenizer in DEPC water tube, switch to "ON", hold for 5 s, and switch to "OFF". Repeat twice. Use Kim wipes to gently dry the tip. Check carefully if any tissue debris gets stuck in the tip.

**Note:** Handheld mechanical homogenizer could be substituted with any other kind of homogenizer. Please follow manufacturer's instructions for the cleaning up steps. Alternatively, if no homogenizer is available, use mortar and pestle instead. To achieve optimal grinding results, put the tissue samples in the mortar, add liquid nitrogen to freeze the samples for a few seconds, and grind. Add ice-cold co-IP buffer and proceed to the next steps.

10. Sonicate the samples at 4°C at a constant full speed for 10 s for 3 times (Figure 5, Branson Sonifier 250, Duty cycle "constant", Output control "4", press "Hold" for 10 s, break 1 min, repeat for 3 times). Ensure that the liquid is clear and no obvious cell clusters are seen.
11. Centrifuge the lysates at 12,000 × g at 4°C for 10 min to get rid of the cell debris.
12. Collect supernatant in a new set of 1.5 mL tubes. Discard the pellets.
13. Determine the protein concentrations by the Bradford protein assay following the manufacturer's instructions using disposable cuvettes and a spectrophotometer at 595 nm wavelength. (? [troubleshooting](#)).
14. Equalize the concentrations of the protein samples in all the experimental groups using co-IP buffer (1% proteinase inhibitor added).

**Note:** Add RNase inhibitors according to manufacturer's guidelines RNase inhibitor in addition to proteinase inhibitor.

15. Save 4% of the total lysates as Input in a new set of 1.5 mL tubes. (? [troubleshooting](#)).
16. Subject the rest of the protein samples directly into the pull-down process.



**Figure 5. Workflow for Part III, IV, V**

Lysates preparation, Pull-down Procedure, and Further Application (Total Timing ~ 6 h). Prepared lysates should be incubated with pre-treated probe (Pre-treat the probe for 95°C 10 min, immediately followed by 0°C ice-water mixture treatment for 5 min) for 2 h at 25°C. The incubated probe-lysates mixture should be subjected to another incubation with washed streptavidin beads for 1 h at 25°C. The probe-lysates-beads mixture should be washed at least 6 times with co-IP buffer and a change to new set of tubes should be ensured at the time of last wash. Further wash should be conducted based on the application needs.

▮▮ **Pause point:** Protein samples can be stored at  $-80^{\circ}\text{C}$  for up to one week.

### Circular RNA pull-down

⌚ **Timing:**  $\sim 4$  h

This section is the pull-down process where circular RNA probe will pulldown the target circular RNA, and further pulldown the binding partners in your interested samples.

#### 17. Pre-treatment of the Probe (Figure 5②):

- a. Aliquot amount of probe from stocking on a  $2\ \mu\text{g}$ - per- $1\ \text{mg}$  total protein base to a  $1.5\ \text{mL}$  tube.
- b. Seal the mouth of the tube with a parafilm to prevent accidental collapse in the subsequent water bath heating step.

**Note:** A thermomixer is substitutable for the heating process. However, water bath incubation is highly recommended to achieve optimal outcomes.

- c. Put the tube in a  $95^{\circ}\text{C}$  water bath for 10 min. Use a tube holder to hold the tube vertical to ensure that the liquid surface is under water bath.
- d. Immediately take the tube out from water bath and put the tube on icing water ( $0^{\circ}\text{C}$ ) for 5 min.

⚠ **CRITICAL:** This step needs to be done very quickly to ensure that the probe stays single-stranded and does not anneal back to form a double-stranded structure.

#### 18. Add the probe into lysates prepared from step 9.

#### 19. Incubate the probe-lysate mixture at $25^{\circ}\text{C}$ on a shaker for 2 h.

#### 20. Bead Washing is performed as follows (Figure 5③):

- a. Take out the desired amount of streptavidin-coupled Dynabeads from  $4^{\circ}\text{C}$  fridge (at a  $50\ \mu\text{L}$ -per- $1\ \text{mg}$  total protein base) and resuspend the beads by brief vortexing for 30 s.
- b. Wash every  $50\ \mu\text{L}$  Dynabeads in  $1.5\ \text{mL}$  tube as follows (proportionally increase the amount of buffer in each of the following steps based on the requirement):
  - i. Briefly centrifuge the tube in a minicentrifuge to ensure the contents stay at the bottom of the tube. Put the tube with beads on a magnetic rack. Let it stand for 2 min to allow the magnetic beads to settle to one side of the tube.
  - ii. Discard the supernatant and resuspend the beads with  $1\ \text{mL}$   $1\times$  Washing buffer. Mix the beads well by tapping the tube.
  - iii. Briefly centrifuge the tube, magnetize the beads and discard the supernatant. Add  $50\ \mu\text{L}$   $1\times$  Washing buffer to the beads and repeat the washing step.
  - iv. Wash the beads with  $50\ \mu\text{L}$  Buffer A twice.
  - v. Wash the beads with  $50\ \mu\text{L}$  Buffer B once.
  - vi. Magnetize the beads, discard the supernatant and resuspend the beads in  $50\ \mu\text{L}$  Buffer B. Beads are ready to be used for further steps.

#### 21. Add washed beads to the probe-lysate mixture in step 19.

#### 22. Mix the beads by inverting the tube for 10 times.

#### 23. Incubate the mixture at $25^{\circ}\text{C}$ for 1 h with gentle shaking.

#### 24. Briefly centrifuge the tube, magnetize the beads and discard the supernatant. Start beads washing.

#### 25. Wash the beads with $200\ \mu\text{L}$ of ice-cold co-IP buffer (1% proteinase inhibitor is NOT required).

#### 26. Briefly centrifuge the tube, magnetize the beads and discard the supernatant.

#### 27. Repeat step 25 and step 26 for 6 more times.

28. Add 200  $\mu\text{L}$  of ice-cold co-IP buffer to the magnetic beads. Pipette it up and down until fully mixed (DO NOT shake the tube to avoid any waste of sample on the tube lid) and transfer the mixed solution into a new tube.

**△ CRITICAL:** Change to a new set of tubes can largely reduce nonspecific binding.

29. Let the tube stand on the magnetic rack for 2 min. Discard the supernatant and the beads are ready to be used for further applications.

### Further application

⌚ Timing: variable

This section is the final step of the pulldown procedure where you will gather results from your pull-down process.

30. Follow Option a or b if one wishes to study the circular RNA-protein interaction. Follow option c if miRNA sponge mechanism or detection of any RNA is the subject.

a. For detection of circRNA pulling down protein binding partners by Mass Spectrometry (LC-MS/MS) analysis, wash the beads twice with PBS.

**△ CRITICAL:** Mass Spectrometry centre usually requires a detergent-free condition. Therefore, PBS wash steps are necessary.

Discard PBS as much as possible. Store the beads in  $-80^{\circ}\text{C}$ . Send the beads out on dry ice for mass spectrometry detection. Alternatively, elute the beads with 50  $\mu\text{L}$  ddH<sub>2</sub>O at  $60^{\circ}\text{C}$  for 10 min. Send the eluted sample for mass spectrometry detection.

b. For detection of the circRNA pulling down specific protein, add 40  $\mu\text{L}$  of 1 × Laemmli sample buffer supplemented with  $\beta$ -mercaptoethanol to the magnetic beads. Heat the samples at  $95^{\circ}\text{C}$  for 10 min and proceed to western blotting.

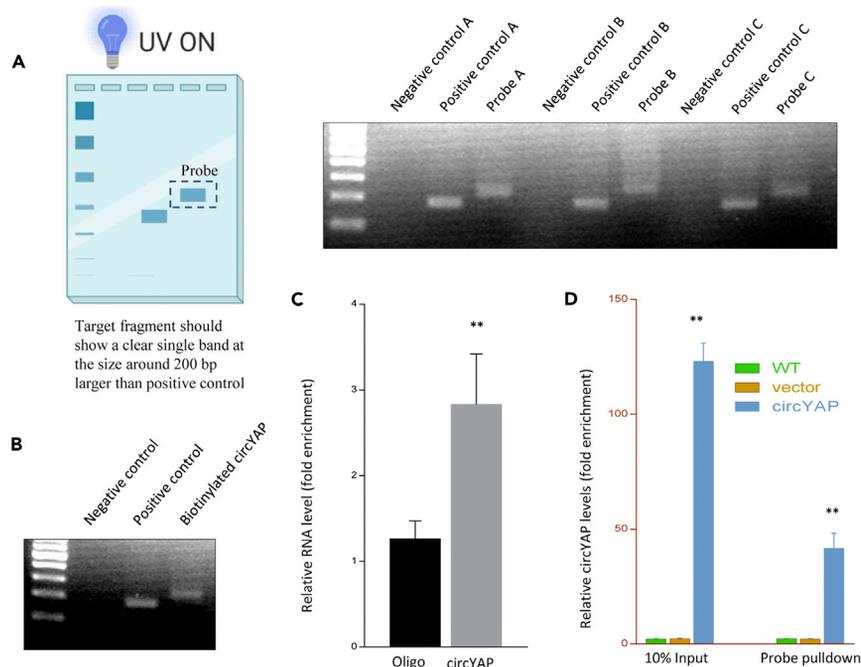
c. For RNA detection, add 300  $\mu\text{L}$  Trizol to the beads. Vortex to ensure maximum elution. Leave on ice for 5 min. Extract RNAs with chloroform-isopropanol RNA precipitation process.

### EXPECTED OUTCOMES

Circular RNA pull-down assay is a powerful technique to uncover potential underlying mechanisms involved in circular RNA functions and could be even more influential when combining it with other applications including mass spectrometry, western blot, and other RNA interference-based strategies. Since further applications following pull-down assays may have many time-consuming, labor-intensive and cost-consuming experimental steps, ensuring the preliminary success of the pull-down is the key to ascertaining the reliability of the results in the subsequent experiments. Such “ensuring” steps include but are not limited to a pre-test of probe condition, and an RNA extraction procedure followed by RT-qPCR to check the expression of the pulled down target circular RNA.

#### Pre-test to seek optimal probe condition

To achieve the best pull-down results, a pre-test of probe condition is highly recommended (i.e., follow steps 1–4). Diagram (Figure 6A, Left) shows the anticipated results that should be observed on a DNA gel after PCR amplification of a circular RNA probe (along with the negative control and the positive control). Due to the biotin labeling process, the target circular RNA probe band should show a clear single band that is slightly larger than the positive control band. The target band should be less bright compared to the positive control band as well (Figure 6A, Right). A representative image of an ethidium bromide stained 2% agarose gel showing the correct probe products of the target circular RNAs is shown in Figure 6A, Right. Three circular RNAs A, B, and C, are



**Figure 6. Anticipated results for probe generation and circular RNA pull-down by the probe**

(A) Left, a diagram showing expected results visualized on gel for probe generation. Right, a representative image showing successful probe generation for three randomly chosen circular RNAs. A correct probe should show a clear but less bright band with a size 100 bp bigger compared to the positive control.

(B) Agarose gel showing amplification of biotinylated circYAP probe.

(C) The biotinylated probe precipitated significantly more endogenous circular RNA in MDA-MB231 cells that express high levels of circYAP.

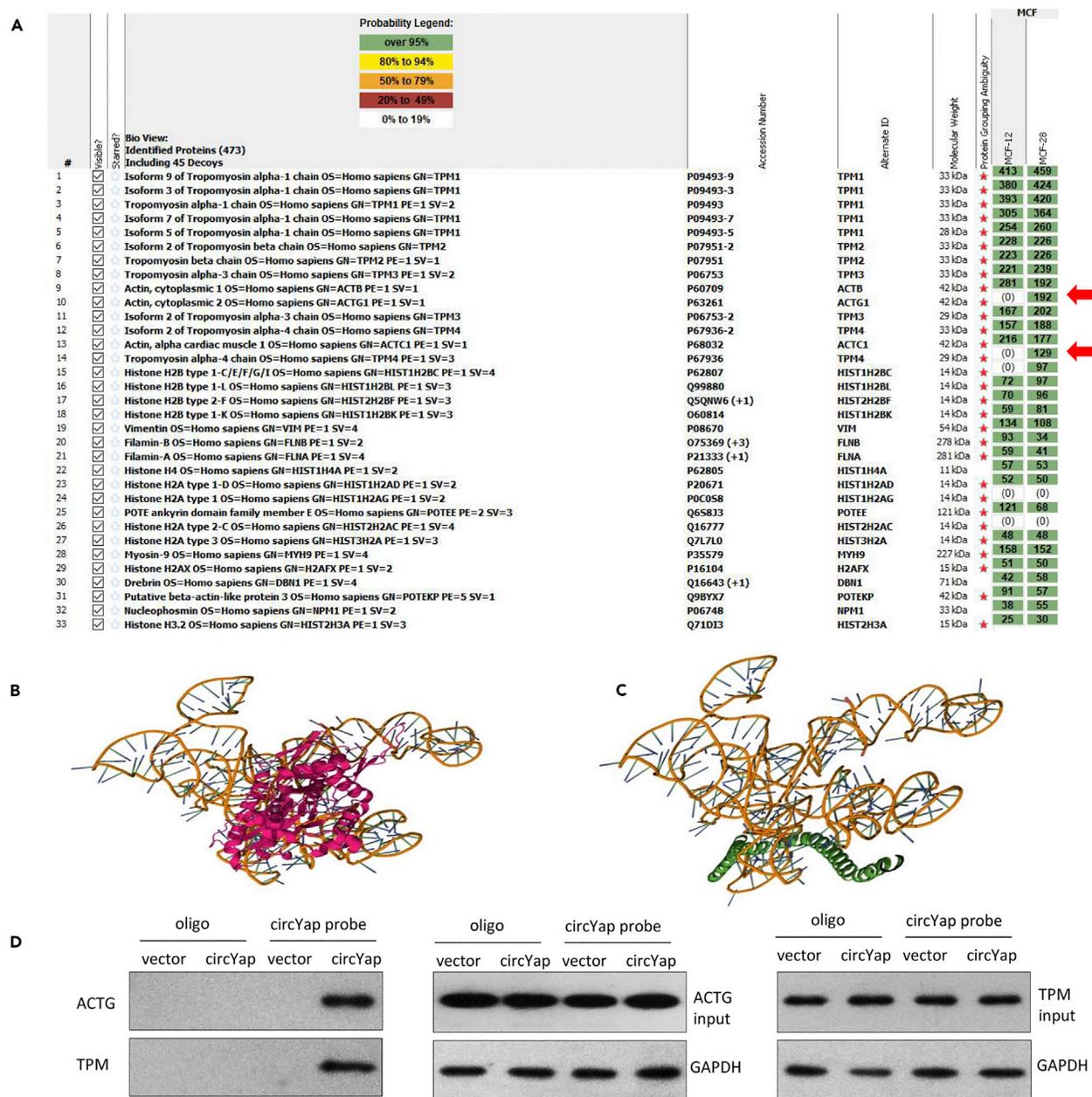
(D) The plasmid circYAP was also expressed in MCF cells that expressed low levels of endogenous circYAP. The probe could also pull down significantly higher levels of circYAP relative to the un-transfected (WT) and vector-transfected cells.

randomly chosen for probe design and probe amplification. Sequences of each circular RNA plasmid and probe are provided in [Table 2](#). This image indicates that our protocol could successfully generate probes for various circular RNA targets. There are multiple factors that potentially affect the generation of probe products and annealing temperature is one of the most important factors. If an unclear/smear band was observed, an alternative PCR condition should be chosen (See [troubleshooting](#) for further information). While 56°C is the most recommended annealing temperature as a start for pre-test, some probes may need a lower temperature to achieve clear products (for example 50°C). Adjust the temperature before any other adjustments like cycle number, since increasing PCR cycles leads to potential risks in generating non-specific products.

#### Anticipated relative RNA level after pulldown process

Following confirmation of successful probe generation, another step that could ensure reliable outcomes for any further application is to save some magnetic beads for RNA extraction and pursue with RT-qPCR analysis to check the relative circular RNA levels between groups. We use the pull-down process for circYAP as an example to illustrate this step. A representative photo of circYAP probe was shown in [Figure 6B](#). Relative circYAP level following RNA pull-down procedure in wild-type cells was shown in [Figure 6C](#). Comparing with the oligo probe, circYAP probe pulled down a significantly increased amount of circYAP in wild-type MDA-MB231 ([Figure 6C](#)). In addition, in the MDA-MB231 lysates which were overexpressed either with vector plasmid control or circYAP plasmid, incubation of circYAP probe in the groups showed more pulled down circYAP in the circYAP plasmid overexpression group compared to the vector control ([Figure 6D](#)).





**Figure 7. Example data showing the results following circular RNA probe pull-down process to uncover mechanism studies**

(A) Representative mass spectrometry analysis showing the binding partners of circYAP (Wu et al., 2021).

(B and C) Analyzed binding sites between circYAP and ACTG (B) or TPM4 (C). Predicted 3-dimensional structures showing the interaction between circYAP and ACTG (B) (Wu et al., 2021) or TPM4 (C) (Wu et al., 2021).

(D) Pull-down assay followed by western blot using specific antibodies confirmed the binding between circYAP and ACTG or TPM4 (Wu et al., 2021).

identification of all proteins that interact with the circRNA of interest if the biotin-RNA pull-down product is subjected to mass spectrometry. The precision and sensitivity of this method allows low abundantly expressed proteins to get enriched (e.g., membrane proteins following subcellular fractionation). Potential bonded proteins could be detected through this pull-down method followed by western blotting to identify specific protein interactions. This pull-down method could be even more powerful in studying circRNA-protein interactions if combined with computational techniques and RNA-interference-based strategies.

Efficiency and specificity are the two most important determinants of the success of this pull-down process. And the potential limitations of this optimized protocol could also lie in the balance of these two compartments. We optimized our protocol by modifying a couple of steps including repeats of a BSJ sequence in the probe, the ratio of forward primer and reverse primer in the probe synthesis process and the probe pre-treatment tips. We demonstrate that with these procedures, it is possible to significantly enrich the predicted targets of circRNAs. However, the four repeats of the probe work best in our experimental conditions and fit for our research goals, it may not be the ideal condition for studies that prefer focusing on one of the major focuses (i.e., prefer efficiency to screen more binding partners or prefer specificity to only target a specific group of proteins). We noted that the traditional synthetic probe containing 5' or 3' biotinylation with no repeat of the probe sequence could specifically pull down the circRNA of interest. However, such a probe had low efficiency in pulling down the circRNA and often showed poor signal for detection. While our four repeat probe contained multiple biotinylation sites that significantly increased the efficiency of the pull-down, the PCR method generated some unwanted reversed sequence. This reversed sequence could potentially pull down non-specific transcripts resulting in decreased specificity of the pull-down product. Thus, if specificity is the major concern, one may want to use the synthetic probes.

This protocol does not necessarily require exogenous circular RNA expression to achieve binding partners detection, however, it is recommended to have overexpression steps to enrich the circular RNAs where the endogenous expression levels of the circRNAs were lower than 0.2 copies per cell. A copy number range from 0.2 – 0.5 per cell is optimal enough while conducting our protocol. A circular RNA with higher endogenous expression level (1–2 copies per cell) should have no issue in direct execution of this protocol without any transfection steps. For researchers that are new to the circular RNA field and interested in circRNA studies, this protocol provides step by step details of the complete procedure from probe designing to the further applications of the pull-down assay products. For researchers that are in the field of circular RNA studies, our protocol provides new insights to their current circRNA pull-down system.

## TROUBLESHOOTING

### Problem 1

Do not observe clear bands for probes as expected (step 4).

### Potential solution

There are a couple of circumstances where expected outcomes may not be observed in the probe generation process. No band in positive control may be due to a failure in the plasmid extraction process. A solution for this could be re-extraction of the plasmid. Make sure that the correct antibiotics are added to the LB medium. Make sure to measure the concentration and quality of the plasmid after extraction. If a band in the negative control was observed, sample contamination could be a possible reason. Please make new control use fresh reagents, especially the ddH<sub>2</sub>O. Use filter tips and change tips more often. If no band was observed in the circular RNA probe product, a possible explanation would be too high of the annealing temperature during PCR synthesize procedure. While our protocol standardized the annealing temperature to 56°C, a lower annealing temperature of 50°C could be used when such an issue is encountered. There are a couple of reasons if an unclear band was observed in the circular RNA probe product. On one hand, it might be due to an insufficient PCR template issue. If the concentration of the plasmid was lower than 200 ng/ul, add more plasmid (template) when synthesizing, adjust the volume using ddH<sub>2</sub>O. On the other hand, it may occur when the annealing temperature was too low for that specific probe. A higher annealing temperature of 60°C could be used to overcome such issue. Last but not the least, if no difference in band sizes was observed between the positive control and the circular RNA probe, a failure of biotinylation of the circular RNA probe could be the issue. Other than non-ideal synthesizing temperatures during PCR procedures as mentioned above, changing to a new biotin CTP could be considered as a potential solution. Another solution for this issue is to use a diluted dNTP set instead of the suggested concentration. Use sterile water

(DNase/RNase-free water) to dilute your original dNTP set to 1:50 or 1:100, and prepare the reaction system following the same setup. Use new biotin CTP to resynthesize the circular RNA probe. Furthermore, if multiple bands of the circular RNA probe were visualized in ultraviolet transilluminator, proceed to purification through gel extraction.

### Problem 2

Wavelength reading was very small (step 13).

### Potential solution

This might be due to the protein concentration being too low and it is not ideal to pursue following steps. Do not pursue following steps if came across this problem. Check confluency of the cells before collection. If the confluency of the cells was good, increase the number of the cell culture plates. This protocol standardized the plate numbers to 2 × 100 mm plates of cells for pull-down followed by western blot and 5 × 100 mm plates of cells for pull-down followed by mass spectrometry analysis. Please increase the plate numbers until the desired protein concentration was achieved based on the specific analysis (check with analysis centre for recommendation guidelines for the minimum concentration). Another possible reason for not detecting an ideal wavelength can be the choice of cells that express lower level of proteins. This issue may be encountered when you are dealing with B16 cell lines. Double check if the cells of choice express acceptable amounts of proteins.

### Problem 3

Input shows no overexpression of circular RNAs (step 15).

### Potential solution

This issue might happen when the transfection procedure failed or transfection efficiency was low. Optimize the transfection conditions. The optimization processes could start from increasing the plasmid concentration (start from doubling the amounts), increase the transfection incubation time (change from 6 h to overnight transfection). Other than optimizing these conditions, check if the cells are mycoplasma free.

### Problem 4

No expression difference was observed in pulled down proteins (steps 30 a & b).

### Potential solution

Increase the incubation time of the probe and the lysates from 2 h at 25°C to overnight at 4°C. Increase the forward primer and the reverse primer to a higher ratio. A recommended ratio could be 1:10.

We provided above troubleshooting advice for most frequently asked scenarios. Based on our experiences in handling different circular RNAs, a successful pull-down result would be achieved if the above troubleshooting steps were checked. If the protocol did not work with all major troubleshooting advices, double check the pH of Buffer A, Buffer B, Washing buffer. The pH of buffers in beads washing steps are critical and would affect the binding affinity. In addition, it should be emphasized that Biotin-16-dCTP could be changed to Biotin-16-dGTP or Biotin-16-dATP or Biotin-16-dTTP. This depends on which nucleobase presents the most in the probe sequence. Moreover, based on our experiences, before and after purification procedures provided similar results in regards to the successful generation of the probe. Therefore, we do not proceed an additional purification step if we observed a clear single product band. However, if multiple bands were visualized in ultraviolet transilluminator, purification through gel extraction should be performed.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Burton B. Yang, [byang@sri.utoronto.ca](mailto:byang@sri.utoronto.ca).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate any unique datasets or code.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101702>.

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## AUTHOR CONTRIBUTIONS

N.W., F.L., W.W.D., and B.B.Y. conceived and optimized the protocols. F.L. wrote the manuscript. F.L., Q.Y., and B.B.Y. revised and edited the manuscript. B.B.Y. supervised the project.

## DECLARATION OF INTERESTS

The authors declare no conflict of interest for the study.

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