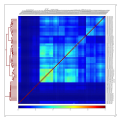

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Working

FLASH

Version 1

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ABSTRACT

Determination of the *in vivo* binding sites of RNA-binding proteins (RBPs) is paramount to understanding their function and how they affect different aspects of gene regulation. With hundreds of RNA-binding proteins identified in human cells, a flexible, high-resolution, high-throughput, highly multiplexible and radioactivity-free method to determine their binding site has not been described to date. Here we report FLASH (Fast Ligation of RNA after some sort of Affinity Purification for High-throughput Sequencing), which uses a special adapter design and an optimized protocol to determine protein-RNA interactions in living cells. The entire FLASH protocol, starting from cells-on-plates to a sequencing library, takes 1.5 days. We demonstrate the flexibility, speed and versatility of FLASH by using it to determine RNA targets of both tagged and endogenously expressed proteins under diverse conditions *in vivo*.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Will be added.

GUIDELINES

This protocol describes the variant XF2. To see all variations of the protocol see Ilik et al., 2019.

MATERIALS

NAME	CATALOG #	VENDOR
T4 RNA Ligase 1 (ssRNA Ligase) - 1,000 units	M0204S	New England Biolabs
Shrimp Alkaline Phosphatase (rSAP) - 500 units	M0371S	New England Biolabs
Magnetic Particle Concentrator (MPC): Dyna-Mag2	12321D	
RNasin(R) RNase Inhibitor, 2,500u	N2111	Promega
NEBNext Ultra II Q5 Master Mix - 250 rxns	M0544L	New England Biolabs
Agencourt Ampure XP	A63880	Beckman Coulter
CircLigase II kit	CL9025K	Epicentre
SUPERase• In™ RNase Inhibitor (20 U/µL)	cat# AM2694	Thermo Fisher Scientific
Flp-In™ T-REx™ 293 Cell Line	R78007	Thermo Fisher Scientific
Dynabeads™ His-Tag Isolation and Pulldown	10103D	Thermo Fisher Scientific
Dynabeads™ MyOne™ Streptavidin C1	65001	Thermo Fisher Scientific
T4 Polynucleotide Kinase	M0201S	New England Biolabs
Proteinase K Solution (20 mg/mL) RNA grade	25530049	Thermo Scientific
SuperScript™ III First-Strand Synthesis System	18080051	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
Phosphate buffered saline (10× concentrate BioPerformance Certified suitable for cell culture)	P5493-1L	Sigma-aldrich
Triton™ X-100	T9284	
TWEEN® 20	P7949	Sigma Aldrich
IGEPAL® CA-630	I8896	
Roti®-Stock 20 % SDS	1057.1	Carl Roth
Sodium chloride	S3014	Sigma Aldrich
Lithium chloride	793620	Sigma Aldrich
Imidazole	I5513	Sigma Aldrich
Trizma® base	93350	Sigma Aldrich
Nuclease-Free Water (not DEPC-Treated)	AM9937	Thermo Fisher Scientific
FastStart Universal SYBR Green Master (Rox)	4913850001	Roche

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Dynabeads™ His-Tag Isolation and Pulldown	10103D	Thermo Fisher Scientific
SuperScript™ III First-Strand Synthesis System	18080051	Thermo Fisher Scientific

MATERIALS TEXT

All buffers are prepared with autoclaved milli-Q water in an RNase-free environment. All buffers are filtered through single-use 0.22µm filters and autoclaved except 2X PNK-MES buffer (pH 6.0) which was only filtered. **Be careful with buffers HSB, 2XNLB and LDS-buffer, risk of overflow during and after autoclaving them!** Let 2XNLB cool down to room temperature and mix the contents by swirling the bottle.

The recipes are below:

2XNLB (dilute to 1X with autoclaved milliQ water, or equivalent)
2X PBS
0.6M NaCl
2% Triton™ X-100
0.2% TWEEN® 20

NDB
50mM Tris.Cl (pH 7.4)
0.1M NaCl
0.1% TWEEN® 20

HSB
50mM Tris.Cl (pH 7.4)
1M NaCl
1% IGEPAL® CA-630
0.1% SDS
1mM EDTA (pH 8.0)

2X PNK-MES buffer (pH 6.0)
50mM MES (pH 6.0)
100mM NaCl
20mM $MgCl_2$

0.2% TWEEN® 20

LDS-buffer
20mM Tris.Cl (pH 7.4)
0.5M LiCl
0.5% LiDS
1mM EDTA (pH 8.0)

PLB
20mM Tris.Cl (pH 7.4)
0.5M LiCl
1% Triton™ X-100
0.1% SDS
1mM EDTA (pH 8.0)

2X ProK buffer
20mM Tris.Cl (pH 7.4)
100mM NaCl
0.2% TWEEN® 20
20mM EDTA (pH 8.0)

SAFETY WARNINGS

UV-C light is dangerous to the naked eye and skin, make sure that the UV-crosslinker functions properly and does not let UV-C light through. Please read the MDS of all the chemicals used in this protocol.

BEFORE STARTING

Read the manuscript, Ilik et al., 2019.

Lysate preparation

1 **Crosslink cells with with UV-C (0.15 - 0.2 mJ/cm2) irradiation.**

Type, amount, state of the cells varies depending on the intended application. As a rule of thumb, crosslink cells in 15cm-plates on water/ice and as close to the lamp as possible (3-6 cm distance). Wash the cells with ~6mL of ice-cold PBS, and add 6mL of ice-cold PBS again before crosslinking. It is important to do at least one wash of PBS to remove UV-absorbing media components (phenol red, nucleotides etc.). We usually use half of one 15cm-plate for one IP/pull-down. In a typical experiment, two 15cm plates are crosslinked, and half of the cells from each plate is used as a biological replicate pair, and the other two are kept as backup. The cells, if they are not used immediately, are spun down at 1000*g* for 1 minute, and snap-frozen with liquid nitrogen after removal of PBS, and kept at -80˚C until use.

2 **Re-suspend the cell pellet with 600 µl 1xNLB , sonicate with Bioruptor Plus (5 cycles, LOW, 00:00:30 ON, 00:00:30 OFF). Spin-down the lysate @ 20.000*g* for 00:10:00 , use the supernatant for IP/pulldown.**

There are a few important points here. NLB (short for Native Lysis Buffer) is used here for cell disruption, because it is a relatively high-salt buffer (~500mM NaCl) with high amounts of non-denaturing detergents (1% Triton-X, 0.1% Tween-20). Combined with the Bioruptor treatment, this homogenization/disruption method is very effective in solubilizing target proteins. It is advisable to check if the target is indeed solubilized properly before proceeding with the rest of the protocol.

Other types of sonication/homogonization may be possible, and must be tested empirically. We do not recommend using Bioruptor Pico for this purpose.

Polyhistidine Purification

3 **Add the lysate to 25µL of His-Tag Isolation and Pulldown beads.**

Take 25µL of nicely resuspended beads (resuspending either by vortexing, or preferably by rotating the beads end-to-end in the cold-room

until no clumps are visible on the walls), wash them once with 1xNLB, resuspend with 500 µl NLB. Add the lysate to this suspension, pipette up and down 2-3 times then incubate for 5-10 minutes on ice. Rotation is not necessary, these beads do not settle in this time-frame. Wash the beads 1x with NLB.

Dynabeads™ His-Tag Isolation and Pulldown
by Thermo Fisher Scientific
Catalog #: 10103D

Many researchers are very sceptical about poly-histidine purifications, because they tend to be “dirty”. This is indeed true, single-step poly-his purifications are generally quite dirty. What we do here however is a bit different: these beads almost quantitatively collect the target protein only after **5 minutes of incubation**. This is quite extraordinary, and coupled to a second purification (via the Biotin group in this protocol) leads to very clean purifications. The speed of the first pull-down probably helps to remove most of the extremely abundant RNAs that tend to be absorbed to bead surfaces during extended purifications.

4 **Elute with 0.5mL NLB + 250mM imidazole, 5 minutes on ice.**

One can use as little as 50mM imidazole, these beads release target very quickly with >50mM imidazole. The elution buffer is prepared using 2xNLB stock, 2M imidazole pH 8.0 and water.

Streptavidin purification

5 **Incubate the eluate with 25µL MyOne™ Streptavidin C1 beads (in 500µL NLB) for 00:45:00 in the cold-room. Wash once with HSB and once with NDB.**

The incubation can be as short as 30 minutes and as long as 2 hrs, this can be empirically determined for each protein. It would be best to limit the incubation to the shortest possible time to reduce non-specific RNA absorption on the beads. Other streptavidin-coupled beads may also be used, however agarose/sepharose beads should best be avoided.

Partial RNase digestion

6 **Re-suspend the beads with 90µL NDB. Add 10µL of RNaseI diluted in NDB (1:500-1:2000). Incubate at 37 °C for 00:03:00 . Keep on ice for a minute. Wash with HSB, then NDB.**

This step is very important. The more RNAse once uses, the sharper the peaks become, but the recovery is also reduced. We have used dilutions ranging from 1:400 to 1:8000. We would recommend to start with 1:2000. Due to batch effects, and other uncontrollable laboratory conditions, it is highly recommended to initially prepare a library where distinct s-oligos are used with different RNAse treated samples, which are then pooled after ligation and sequenced.

Dephosphorylation of RNA-ends with T4 PNK

7 **Resuspend with 20µL of the MES-PNK mix. Incubate @ 37˚C for 20 minutes. Wash once with HSB, once with NDB.**

At this step, T4 PNK is used to dephosphorylate the 3’-ends of RNAseI-digested RNA. RNaseI leaves a cyclic 2’,3’-bisphosphate behind, which is refractory to adapter ligation. T4 PNK removes this and other phosphate groups and leaves a 3’-OH, which is suitable for ligation. NEB also sells a phosphatase-minus PNK, obviously do not use that. Any other RNase-inhibitor would work here, and addition of β-mercaptoethanol is absolutely necessary.

2X PNK-MES buffer (pH 6.0)	10µL
SUPERase• In™ (20U/µL)	0.5µL
β-mercaptoethanol (0.1M)	1µL
T4 PNK (10U/µL)	1µL
H_2O	7.5µL

T4 PNK dephosphorylation master mix

s-oligo ligation

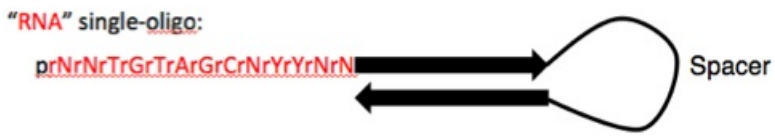
8 **Wash once with HSB, merge all relevant samples. Wash once with LDS-buffer, once with PLB buffer, 1x HSB, 1x NDB.**

Carry out LDS and PLB washes at room-temperature, and the rest in the cold-room or on ice.

Stringent washes

9 **Re-suspend the beads with 7µL of room-temperature, RNase-free water. Add 1µL of selected s-oligo (10µM). Add the rest of the ligation mix. Incubate @ 25˚C for 60 minutes**

1. PEG8000 and ATP is shipped with T4 RNA Ligase, when ordered from NEB. PEG8000 is extremely viscous, and that is the reason why the reaction is set up in this way (first water, then adapter, then the rest).
2. Adapters are internally barcoded (TGTAGC below). It is possible to mix negative controls (GFP-expressing cell line, parental cell line, point mutant that is supposed to not bind RNA etc) with the sample after ligation. This allows us to evaluate background very precisely and filter false-positives efficiently.
3. The adapters are key to the success of this protocol. They basically look like this:



This adapter is ligated to RNA via T4 RNA ligase. The self-annealed part then is used as a reverse-transcription primer. At the end of reverse transcription, RNaseH is used to remove RNA and create a 5'-phosphate at the edge of the adapter. This cDNA molecule is then circularized by CircLigase (requires 5'-phosphate) and then PCR-amplified (adapter is based on the Y-shape adapter of Illumina and contains P5 and P3 sequence for amplification). We have a patent application for it too: WO/2017/013005

10X T4 Ligase Buffer	2µL
PEG8000	4µL
s-oligo (10µM)	1µL
Superas-IN	0.5µL
T4 RNA Ligase 1 (10U/µL)	1µL
ATP (1mM)	2µL
H_2O	7 +**2.5**µL

T4 RNA Ligase 1 Master Mix

Removal of the 3'-phosphate from s-oligo

10 **Resuspend the beads with 35µL of NDB, add 1µL RNAsin+, 4µL rSAP. Incubate @ 37˚C for 15 minutes. Wash once with HSB, once with NDB.**

This step is necessary to remove a blocking 3'-phosphate from our s-oligo. It is there to prevent self-circularization during ligation, but has to be removed before reverse-transcription. One can also use T4 PNK, exactly as done in step (7).

Deproteinization and recovery of RNA

11 **Resuspend the beads with the proteinase-K mix and incubate for 20 minutes at 42˚C.**

2X ProK buffer	50µL
20% SDS	1µL
Proteinase K (20mg/mL)	10µL

H_2O	39µL

Proteinase-K mix

12 **Magnetize the beads, remove the supernatant and transfer to a new tube. Use the Oligo Clean and Concentrator Kit to purify RNA (use 200µL Binding Buffer and 400µL of Ethanol for binding). Elute with 10.5µL of RNase-free water.**

Reverse transcription and phosphorylation of 5'-ends

13 **Incubate the RNA elution at 65 °C fro 2 minutes, transfer immediatately on ice, leave for a minute. Add the rest of the reverse-transcription mix, prepared as per SuperScript III manual. Incubate at 42˚C, then at 50˚C, then at 55˚C, 10 minutes each.**

SuperScript™ III First-Strand Synthesis System
by Thermo Fisher Scientific
Catalog #: 18080051

10X RT Buffer	2µL
10mM dNTPs	1µL
25mM $MgCl_2$	4µL
0.1M DTT	2µL
RNaseOUT (40U/µL)	0.5µL
SSIII (200U/µL)	0.5µL
RNA eluate in H_2O	**10**µL

14 **In order to remove RNA and phosphorylate the 5'-ends, cool the RT reactions to room-temperature, and add 2µL of *E. coli* RNaseH (2U/µL), which is shipped with the SuperScript III First-Strand Synthesis System. Incubate for 00:20:00 at 37 °C .**

Other sources of RNaseH can be used as long as it is an RNaseH1-type, processive RNaseH and not the RNaseH2-type which is not processive. Alternatively, RNA can be removed with NaOH, followed by T4 PNK-mediated phosphorylation of the cDNA ends. See the manuscript for details.

15 ***Optional:* To remove any RNA that may be left in the reaction, add 2.5µL of 1N NaOH. Incubate @ 80˚C for 5 minutes. Neutralize with 25µL 0.2M Tris.**

16 **Use the Oligo Clean and Concentrator Kit to purify cDNA (If step 15 was taken, use 100µL Binding Buffer and 200µL of Ethanol for binding, otherwise, add 28µL of water to the RT reactions first, then continue with 100µL of Binding Buffer). Elute with 6.5µL of water.**

Circularization of cDNA

17 **Set up the circularization reaction. Incubate at 60 °C for at least 02:00:00 .**

10X Buffer	1µL
50mM MnCl2	0.5µL
5M Betaine	2µL
CircLigaseII	0.5µL
cDNA	6µL
	10µL total.

CircLigaseII master-mix

CircLigase can also be used instead of CircLigaseII, remember to add ATP to the reaction!

qPCR to determine optimal cycling conditions

18 **In order to determine the number of cycles we should use to amplify the circularized cDNA, we first set up a qPCR reaction using 1µL of the CircLigase reaction.**

This step is highly empirical and is not meant to be used as a way to quantitate different libraries. The purpose here is to estimate a cycle number with which we are reasonably sure that we are not over- or underamplifying our libraries.

FastStart Universal SYBR Green Master Mix (2X)	10µL
circularized cDNA	1µL
primerL (10µM):	2µL
primerR (10µM):	2µL
Water	5µL

PrimerL (410a in the manuscript): TACACGACGCTCTTCCGATCT
PrimerR (410b in the manuscript): GACGTGTGCTCTTCCGATCT

Library amplification and clean-up with AMPure XP beads

19 **Based on the qPCR results, determine a cycle number that will be used for PCR. In our laboratory setting, Ct-1 generally gives good results. We then use the rest of the CircLigaseII reaction to amplify our library using NEBNext® Ultra™ II Q5® Master Mix using the reaction mixture below.**

The choice of amplification primers depends on the final sequencing platform and type of run that will be carried out. Compatible primer sets include, but not restricted to: NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) (E7600), NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1). Compatibility can be verified by cross-checking the s-oligo sequencing to the primers that will be used.

NEBNext® Ultra™ II Q5® Master Mix (2X)	10µL
circularized cDNA	9µL
P5/i5 (10µM):	1µL
P3/i7 (10µM):	1µL
Water	19µL

Library amplification mix

20 **Clean up the reaction twice with 1.5X AMPure XP beads.**

Add 60µL of beads to the reaction, incubate for 5 minutes. Magnetize the beads and remove the supernatant **carefully**. Wash twice with 500µL of freshly-prepared 70% Ethanol without resuspending the beads or removing the tubes from the magnet. Air-dry for 1-2 minutes and elute the bound DNA by resuspending the beads with 40µL of 10mM Tris pH 8.0, 0.05% Tween-20. Incubate the beads at room temperature for 1-2 minutes. Magentize the beads, transfer the eluate to a fresh tube, and repeat the purification as described above. Elute the final library with 10-20µL of 10mM Tris pH 8.0, 0.05% Tween-20.

Library quantification

21 **Quantify the end product using Qubit or another sensitive dsDNA quantification platform. It is also generally necessary to determine the size-distribution of the library, which can be carried out with an Agilent Bioanalyzer 2100, or Fragment Analyzer or an equivalent system. Ready for sequencing!**

After sequencing, the UMI and the index can be found in the first 13 nucleotides of the R2 reads with this pattern: NNNNNXXXXXXNN, where XXXXXX is the index and the Ns together form the UMI.

See https://clipseq.usegalaxy.eu/ for a quick introduction to CLIP-data analysis.