microbial iCLIP2 protocol

Intention:

- This step-by-step protocol is based on the original iCLIP2 methodology **(Buchbender et al., 2020).** Specific adaptations tailored to the use with filamentous cells of the fungal microorganism *Ustilago maydis* were introduced, particularly in the cell lysis procedure. Note (Buchbender et al., 2020) provide additional tips and detailed technical guidance that should be consulted**.** For further information or assistance, contact Nina.Stoffel@hhu.de. Note, that the full microbial iCLIP2 procedure will require at least 5 days for completion, including sample preparation, immunoprecipitation, RNA isolation, cDNA synthesis, and library preparation.

Preparations:

- Ensure that all pipettes and materials are thoroughly cleaned and free of RNases
- It is highly recommended to prepare small, single-use aliquots of buffers, primers, and other reagents
- Barcode adapters should be pre-tested using the same input material to ensure consistent and comparable efficiency

Buffer:

Note: *After buffer preparation, it is highly recommended to divide the solution into smaller aliquots (e.g. 50 mL RNase-free tubes) to minimize the risk of contamination. For optimal results, use each aliquot only once.*
Store buffers at 4 °C.

Buffer		
iCLIP2 lysis buffer: 50 mM Tris–HCl, pH 7.4 100 mM NaCl 1% (v/v) IGEPAL CA-630 0.1% (w/v) SDS 0.5% (w/v) sodium deoxycholate 2 M urea **Add inhibitors directly before usage (per 2 mL):** 2× Complete protease inhibitor (Merck, 11873580001) 1 mM DTT 1 mM PMSF (optional: + 5 µl SUPERase-In, Thermo Fisher, AM2696)	**High salt buffer:** 50 mM Tris–HCl, pH 7.4 1 M NaCl 1 mM EDTA, pH 8.0 1% (v/v) Igepal CA-630 0.1% (w/v) SDS 0.5% (w/v) sodium deoxycholate	**PNK wash buffer:** 20 mM Tris-HCl, pH 7.4 10 mM $MgCl_2$ 0.2% (v/v) Tween-20
5× PNK buffer, pH 6.5: 350 mM M Tris-HCl, pH 6.5 50 mM $MgCl_2$ 5 mM DTT - freeze single-use aliquots (200 µL)	**4× Ligation buffer:** 200 mM Tris-HCl, pH 7.8 40 mM $MgCl_2$ 4 mM DTT - freeze single-use aliquots (200 µL)	**Proteinase K (PK) buffer:** 100 mM Tris–HCl, pH 7.4 50 mM NaCl 10 mM EDTA
PK + urea buffer: 100 mM Tris–HCl, pH 7.4 50 mM NaCl 10 mM EDTA 7 M urea	**10x PBS pH 7.4:** 1.37 M NaCl 27 mM KCl 100 mM Na_2HPO_4 18 mM KH_2PO_4	

Cultivation, harvesting, and UV-crosslinking

- Grow a 150 mL culture (OD_{600} = 0.5) in complete medium (CM) supplemented with 1% (w/v) glucose at 28°C with agitation at 200 rpm (see Brachmann et al., 2001)
- Induce filamentous growth by shifting the cells to nitrate minimal (NM) medium supplemented with 1% (w/v) glucose at 28°C, 200 rpm for 6 hours for filament induction (see Brachmann et al., 2001)
- After 6 hours of cultivation, divide the 150 mL culture into three 50 mL tubes
- Harvest the filaments by centrifugation (7,150 x g, 4°C, 10 min) and wash the cells carefully with 5 mL PBS
- Harvest again by centrifugation (7,150 x g, 4°C, 10 min)
- Resuspend the pellets again in 5 mL in PBS
- Transfer cell suspension in a pre-cooled (ice) Petri dish (100 x 100 x 20 mm, Sarstedt: 82.9923.422)
- Place the Petri dish carefully on ice
- (*Tip: to ensure an even distribution of the culture within the Petri dish during UV radiation, the ice is flattened to achieve a uniform plane surface)*
- UV irradiate the cells (254 nm, 1x 200 mJ/cm^2, Biolink UV-Crosslinker, Vilber-Lourmat)
- *(Tip: optimal UV-crosslinking conditions have to be tested for each RBP individual to ensure high quality)*
- Pool the UV-treated cells in a pre-cooled 50 mL tube and place on ice
- Harvest by centrifugation (7,150 x g, 4°C, 10 min)
- Resuspended the pellet in 13 mL PBS
- Divide the homogenous (!) cell suspension into six 2 mL reaction tubes (corresponds to ≈ 25 mL original culture per tube)
- Subsequently, centrifuge at 16,200 x g, 4°C, for 5 min
- Soak the supernatant completely, and eventually, shortly centrifuge again to efficiently remove any residual liquid.
- Place the tubes in a floating holder and flash-freeze the cell pellets in liquid nitrogen
- (*Caution: Handle liquid nitrogen very carefully. Always wear lab goggles, and ensure no liquid nitrogen enters the tubes to prevent an explosion!!!*)
- Tubes can be stored at - 80 °C until proceeding to the next step

Cell lysis

- Add one steel bead (5 mm diameter) to each tube and place the tubes into a pre-cooled TissueLyser Adapter (Qiagen)
- Crack the frozen cell pellets by two rounds of 30 seconds at 30 Hz, with a liquid nitrogen cooling step (30 sec) in between (TissueLyser, Qiagen)
- Add 1 mL lysis buffer (incl. freshly added inhibitors) to the cryogenic cell powder
- For optimal thawing, place the tubes in a Vibrax (Ika-Vibrax-Vxr, Electronic), and resuspend through gently mixing for 5 min, use < 500 rpm at 4°C
- Inspect the lysate, the cell extract needs to be homogenous for proceeding with the next steps
- Separate cell debris by centrifugation at 16,200 x g for 10 min at 4°C

- Transfer the supernatant to a new pre-cooled tube and centrifuge again (16,200 x g, 5 min, 4°C)
- Pool the supernatants in a pre-cooled 50 mL tube. Keep on ice!
- Determine protein concentrations of this cell debris-free supernatant by using a Pierce™ BCA Protein Assay Kit (Thermo Fisher, 23225) and adjust the concentrations to match the sample with the lowest concentration (aiming for ≈ 2 mg/mL)
- (*Note: You can also explore alternative methods for protein quantification. However, ensure that the lysis buffer components, such as SDS or urea, do not interfere with the accuracy of the measurements)*

Immunoprecipitation

Note*: When planning comparative experiments that require multiple batches of affinity beads, it is advisable to pool the beads in advance. This helps to minimize batch effects and ensures consistency across samples*

- Take 60 µL pre-mixed beads (Chromotek; gtma-20; or alternatives) and pre-wash them twice with lysis buffer (without inhibitors)
- Attract the beads using a magnetic separation rack
- For accurate washing, incubate the beads for at least 2 min under continuous rotation
- Split into three fresh pre-cooled 1.5 mL tubes, attract the beads, and discard the buffer
- Distribute the cell debris-free supernatant equally onto the beads
- Immunoprecipitate the samples for 1 hour at 4°C with continuous rotation
- Pool the beads in a 2 mL reaction tube

Optional: RNase I treatment

Note: *This step is only required in case it is needed. Deepening on the RBP of interest. If this does not apply, directly proceed with washing*

- Perform optional RNase I (Life Technologies, AM2295) treatment by testing various dilutions (ranging from 1:50 to 1:1500 in PNK buffer)
- Add the RNase dilution to the beads
- Incubate for 3 min (exactly!!!), 37°C at 1,000 rpm
- Subsequently, directly wash with cold high-salt buffer and handle on ice (to prevent over-digestion)

- Wash four times with 1 mL high-salt buffer
- Wash twice with 1 mL PNK buffer
- Transfer the beads into a fresh pre-cooled 1.5 mL reaction tube
- Wash three times with 1 mL PNK buffer

3’ dephosphorylation and linker ligation

- Soak the supernatant completely
- Resuspend the beads in 20 µl dephosphorylation reaction solution:
    - 15 µL water
    - 4 µL 5x PNK buffer pH 6.5
    - 0.5 µl RNase Inhibitor (SUPERase-In, Thermo Fisher AM2696)
    - 0.5 µL T4 PNK (NEB, M0201S)
- Incubate in a thermomixer at 1,100 rpm and 37 °C for 20 min

- Place the tube on ice and continue under cold conditions
- Attract beads and discard supernatant and wash the beads once with 1 mL 1x PNK
- Wash twice with 1 mL of high-salt buffer
- Wash three times with 1 mL PNK buffer
- Transfer the beads into a fresh 1.5 mL reaction tube and discard the buffer completely
- Link the 20 µL 3' adapter mix to the beads:
    - 8 µL water
    - 5 µL 4x Ligation buffer
    - 0.5 µL RNase Inhibitor (SUPERase-In, Thermo Fisher AM2696)
    - 1.5 µL pre-adenylated L3-App (20 µM)
    - 4 µL PEG400 (Merck, 202398-500G)
    - 1 µL T4 RNA Ligase 1 (NEB, M204L)
- Ensure that the beads remain evenly suspended
- Incubate overnight in a thermomixer at 1,100 rpm and 16 °C
- Attract beads and discard supernatant
- Wash twice with 1 mL of high-salt buffer
- Wash four times with 1 mL PNK buffer
- Transfer the beads into a fresh pre-cooled 1.5 mL reaction tube
- Keep on ice

    Optional: If required, reserve 5% of the sample for Western blot analysis to verify protein presence and quality

5' radioactive labeling (radiation-controlled area !!!)

Notes: *Labeling intensity can vary depending on the sample. For Rrm4, labeling 30-50% of the sample is recommended, while for Grp1, 20-30% is sufficient to achieve reliable results. Reducing the amount of radioactivity is advisable not only for safety reasons but also to minimize radiation-induced RNA damage.*
When working with low-abundance proteins, labeling the complete sample may be necessary. The optimal labeling percentage depends on several factors, including UV crosslinking efficiency, protein abundance, and immunoprecipitation efficiency. Adjustments should be made accordingly to ensure optimal outcomes see **Buchbender *et al.* (2020)**.
Caution: *The general safety measures for work with radioactivity apply!*

- Place the respective amount in a fresh pre-cooled 1.5 mL tube and completely remove the supernatant
- Add 4 µL radioactive labeling mix to the beads and mix carefully
    - 3 µL water
    - 0.4 µL 10x PNK buffer (NEB, M0201S)
    - 0.4 µL ^{32}P-g-ATP (Hartmann, SRP-301)
    - 0.2 µL T4-PNK (NEB, M0201S)
- Incubate in a thermomixer at 1,100 rpm and 37 °C for 5 min
- Remove the supernatant carefully (caution, contains radioactivity!)
- Wash beads twice with 1 mL 1 x PNK
- Add the non-radioactive beads
- Soak the supernatant completely

- Resuspend the beads in 20 µl NUPAGE sample buffer (containing DTT; Thermo Fisher, NP0007)
- Incubate in a thermomixer at 1,100 rpm and 70 °C for 5 min
- Attract the beads and transfer the supernatant into a fresh 1.5 mL tube
- Measure and record the radioactive signal

SDS-PAGE and blotting

- Load the complete sample on a 4-12% NuPAGE-Bis-Tris gel (Invitrogen, NP0321BOX) and use MOPS-running buffer (Invitrogen)
- Use 3 µL pre-stained protein PageRuler™ (Plus) Prestained Protein Ladder (Thermo Fisher)
  Note: Whenever possible, leave at least one empty well between samples to minimize the risk of cross-contamination/lane spilling and to facilitate the excision of the respective areas from the membrane later on
- Let the gel run for 1-1.30 h at 180 V (should be adjusted to the molecular weight of the protein of interest)
- Carefully remove the dye front (radioactive!) and collect the buffer in a canister (slightly radioactive)
- Blot the gel containing the RNA-protein complexes to a nitrocellulose membrane (0.45 µm, Protran Amersham, GE10600033)
- Use NuPAGE transfer buffer (Invitrogen, NP0006) and add 15% (v/v) methanol and the Novex gel electrophoresis apparatus (Thermo Fisher) according to the manufacturer's description
- Blot 1.5 h at 30 V (depending on the MW of the protein of interest)
- *Note: Pre-soak the blotting sponges in the transfer buffer during the SDS-PAGE run to remove as many air bubbles as possible, ensuring efficient and uniform protein transfer)*
- Wash the membrane in PBS
- Cover the membrane with a transparent plastic bag or saran foil
- Expose the covered membrane to a BAS Storage Phosphor Screen (GE Healthcare) for approximately 1-2 hours or overnight at - 20°C (depending on the signal strength and half-time the radioactivity)
- Visualize the screen with a Typhoon™ laser scanner (Cytiva), according to the manufacturer's instructions

RNA recovery and reverse transcription

- The area of interest is 20-60 kDa above the size of the full-length protein: cut the area carefully with a sharp scalpel
- *Notes: clean the scalpel thoroughly between samples to prevent cross-contamination, or ideally, use a fresh scalpel for each sample.*
- *During protocol optimization, cut the membrane into two or three sections—small, medium, and large—to assess if the RNA distribution behaves as expected across different fragment sizes.*
- Place the cut membrane piece(s) into a fresh 1.5 mL tube
- Cut an additional piece from a similar area of one of the free wells as a negative control for all further steps
- Re-expose the membrane to see if the correct area was cut
- Add 200 µL PK-buffer and 10 µL proteinase K (Roche, 03115828001)

- Make sure that the membrane is completely covered
- Incubate at 1,100 rpm and 37 °C for 20 min
- Add 200 µl of PK buffer including 7 M urea
- Incubate at 1,100 rpm and 37 °C for 20 min
- Collect the solution and transfer it to a 2 ml Phase Lock Gel tube (713-2536, VWR)
- Add 400 µl phenol/chloroform (Sigma Aldrich, P3803)
- Incubate at 1,100 rpm and 30 °C for 5 min (do not vortex!!!)
- Spin at 16,200 x g, RT, for 5 min
- Transfer the aquatic phase to a fresh 1.5 mL tube
- Spin at 16,200 x g, RT, for 1 min
- Transfer the supernatant to a fresh 1.5 mL tube
- Add 1 ml 100 % ethanol,1 µL glycoblue (Invitrogen, AM9510), and 40 µl 3 M sodium acetate pH 5.5 (Invitrogen)
- Invert the tube several times
- Precipitate overnight at -20°C
- Spin at 16,200 x g and 4°C, for 30 min
- Remove the supernatant completely
- Wash the pellet with 1 mL 80% ethanol and spin again for 5 min
- Completely remove supernatant (eventually spin again to ensure efficient liquid removal)
- Let the pellet air-dry for 1 min
- Resuspend the pellet in 5 µl RNase-free water by carefully pipetting up and down until the small pellet is completely solved
- Transfer the RNA to a fresh PCR tube
- For the first step of the reverse transcription procedure add:
    - 1 µl primer RT_iCLIP2 (0.5 pmol/µl)
    - 1 µl dNTPs (10 mM)
- Incubate for the first part of the RT procedure:
    - 70°C, 5 min
    - 25°C, hold until the RT mix is added

- Add the RT and mix carefully by pipetting:
    - 7 µl water
    - 4 µl 5x RT buffer (Invitrogen, 18080044)
    - 1 µl 0.1 M DTT (Invitrogen, 18080044)
    - 0.5 µl RNase Inhibitor (SUPERase-In, Thermo Fisher AM2696)
    - 0.5 µl Superscript III (Invitrogen, 18080044)

- Incubate for the second step of the RT procedure:
    - 25°C, 5 min
    - 42°C, 20 min
    - 50°C, 40 min
    - 80°C, 5 min
    - 4°C, hold

- Add 1.65 µl 1 M NaOH and incubate at 98°C for 20 minutes
- Add 20 µl of 1 M Hepes-NaOH pH 7.3

MyONE Silane clean-up (#1)

- Make sure that the MyONE™ Silane beads (Life Technologies, 37002D) are homogenous before pipetting
- Take 10 µL MyONE™ Silane beads per sample
- Attract by magnetic separation rack and soak off the supernatant completely
- Wash the beads with 500 µL RLT buffer (Qiagen, 79216)
- Resuspend the beads in 125 µL RLT
- Add the pre-washed beads to the cDNA library
- Add 150 µL 100% ethanol and carefully mix by pipetting
- Incubate 5 min at RT
- Mix again by pipetting
- Incubate again for 5 min at RT
- Remove the supernatant and wash the beads in 1 mL 80% (v/v) ethanol
- Transfer the mix to a new 1.5 mL tube
- Add 1 mL 80% (v/v) ethanol and incubate for 30 sec at RT
- Attract beads and discard supernatant
- Wash again with 1 mL 80% (v/v) ethanol
- Attract again and let the beads air-dry for 5 min at RT
- Measure your samples and when no radioactive signal is detectable you can leave the radiation-controlled area

Barcode adapter ligation

- Add 2 µL of barcode adapter L#_iCLIP2.0 oligo (10 µM)
    - *Note: assign a unique barcode adapter to each sample to ensure accurate demultiplexing and reliable downstream analysis*
- Add 1 µL 100% DMSO (NEB) to the beads and mix carefully
- Incubate for 2 min at 75 °C
- Briefly spin down
- Immediately place the suspension on ice for < 1 min
- Add 12 µL of the master mix (prepare on ice, ATP is sensitive)
    - 9 µL 50 % PEG 8000 (NEB, M0437M)
    - 0.3 µL water
    - 2 µL 10x NEB RNA ligase buffer (incl. DTT; NEB, M0437M)
    - 0.2 µL ATP (100 mM)
    - 0.5 µL high-concentrated RNA ligase (NEB, M0437M)
- Make sure that the suspension is well-mixed and homogenous
- Add 1 µL high-concentrated RNA ligase (NEB, M0437M)
- Incubate overnight at 1,100 rpm and RT

MyONE Silane clean-up (#2)

- Attract 5 µl fresh MyONE Silane beads (homogenous) to a fresh tube
- Discard the supernatant and wash with 500 µl RLT buffer (Qiagen, 79216)
- Attract beads, discard supernatant, and resuspend the beads in 60 µl RLT buffer
- Add the fresh bead suspension to the bead-cDNA suspension sample from the overnight incubation
- Add 60 µL 100% ethanol, mix by pipetting

- Incubate at RT for 5 min
- Mix it again by pipetting
- Incubate further 5 min at RT
- Attract the beads and discard the supernatant
- Resuspend the beads in 900 µl 80% ethanol and transfer them to a fresh tube
- Attract Settle the beads and discard the supernatant
- Wash with 900 µl 80% ethanol, incubate at RT for 30 sec
- Repeat the washing step for three times
- Attract the beads and discard the supernatant, then air-dry the beads at RT for 5 min
- Resuspend in 23 µl RNase-free water and incubate at RT for 5 min
- Attract the beads and transfer the supernatant to a fresh PCR tube

cDNA pre-amplification

- Add 27.5 µL of the cDNA master mix:
- 25 µL 2× Phusion HF PCR MasterMix (NEB, M0531L)
- 2.5 µL Primer mix of P5_Solexa_S and P3_Solexa_S (10 µM each)
- PCR program :

98 °C	30 sec	
98 °C	10 sec	⎫
65 °C	30 sec	⎬ 6
72 °C	30 sec	⎭
72 °C	3 min	
16 °C	hold	

ProNex size selection (#1)

- Equilibrate the ProNex chemistry (Promega, NG2001) at RT (30 min before use)
- Prepare size selection control (50 µL):
  - 24 µL water
  - 25 µL 2× Phusion PCR MasterMix (NEB, M0531L)
  - 1 µL ULR Ladder (Life Technologies, SM1211)
- Add 147.5 µl ProNex beads (homogenous) per sample into a fresh 1.5 mL tube and add your sample (50 µL) or size selection control (50 µL)
- Pipetting up and down at least ten times (homogenous suspension)
- Incubate at RT for 10 min
- Attract the beads (2 min) and discard the supernatant
- Leave the beads on the stand (!!!!) and add 300 µl ProNex Wash Buffer (Promega, NG2001)
  Note: ensure that the beads are fully covered in wash buffer; if necessary, increase the volume for optimal washing efficiency
- Incubate at RT for 1 min and discard the supernatant (carefully without losing beads)
- Repeat washing once
- Discard the supernatant and let the beads air-dry at RT for 10 min (until cracking is visible)
- Remove the beads from the magnetic rack and resuspend them in 23 µL water (size selection control in 50 µL water)
- Incubate at RT for 5 min

- Attract the beads (2 min) and transfer the supernatant to a fresh tube
- Verify the ProNex size selection efficiency by assessing the size selection control using one of the following methods, as per the manufacturer's instructions:
    - TapeStation (Agilent Technologies, 5067–5584, 5067–5585)
    - Fragment Analyzer (Agilent Technologies, DNF-474)
    - 6% Novex™ TBE gel (Invitrogen, EC6265BOX)
    - Or equal machines/ techniques with high sensitivity
- After size selection, the bands corresponding to 100 bp, 75 bp, 50 bp, and 35 bp should be reduced compared to untreated ULR, indicating successful removal of small fragments. This is detailed in **Buchbender *et al*., (2020)**.

Optimization-PCR

*Note: Determining the optimal number of PCR cycles is crucial for generating high-quality sequencing libraries. Minimizing the number of cycles reduces duplicate reads, thereby increasing the library's complexity, which is essential for reliable and accurate downstream analyses. The optimal number of cycles is 6-11 (*Buchbender *et al.,* 2020*).*

- PCR mix:
    - 3.5 µL water
    - 5 µL 2× Phusion HF PCR MasterMix (NEB, M0531L)
    - 0.5 µL Primer mix of P5_Solexa and P3_Solexa (10 µM each)
    - 1 µL cDNA / control

- PCR program :

98 °C	30 sec	
98 °C	10 sec	⎫
65 °C	30 sec	⎬ 6-11
72 °C	30 sec	⎭
72 °C	3 min	
16 °C	hold	

- Run 2 µL of the PCR product TapeStation, Fragment Analyzer, or (use 8 µL) 6% Novex™ TBE gel

Preparative PCR and multiplexing of samples

- For preparative library preparation, it is recommended to use one cycle less than the previously determined optimal conditions to further minimize duplicate reads and enhance library diversity
- The purified cDNA sample (post-size selection) should be split into two aliquots—one for immediate use and one as a backup.

- Prepare preparative-PCR:
  - 8 µL water
  - 20 µL 2× Phusion HF PCR MasterMix (NEB, M0531L)
  - 2 µL Primer mix of P5_Solexa and P3_Solexa (10 µM each)
  - 10 µL cDNA / control

- PCR program :

98 °C	30 sec	
98 °C	10 sec	X-1
65 °C	30 sec	
72 °C	30 sec	
72 °C	3 min	
16 °C	hold	

- Run 2 µL of the PCR product on a TapeStation or Fragment Analyzer and determine the concentration under the peak
- *Note: Avoid using 6% Novex™ TBE gels for preparative PCR and final library quality control. Use alternative methods like TapeStation or Bioanalyzer for more accurate and reliable quality assessment*
- When the results are adequate (see **Buchbender *et al.*, 2020**), repeat the PCR with the second half of the cDNA library and pool both samples afterward
- For multiplexing, combine different samples (e.g., biological replicates, different proteins) in equal molar concentrations to ensure balanced representation in the sequencing library

ProNex size selection (#2)

- Equilibrate the ProNex chemistry (Promega, NG2001) at RT, 30 min before using
- Prepare size selection control (50 µL):
  - 24 µL water
  - 25 µL 2× Phusion PCR MasterMix (NEB, M0531L)
  - 1 µL ULR Ladder (Life Technologies, SM1211)
- Based on the optimal sample-to-ProNex ratio of 1:2.4 (v/v, see **Buchbender *et al.*, 2020**), calculate and add the appropriate volume of beads to your sample
  (For example: 50 µl sample, add 120 µl of beads)
- Pipetting up and down at least ten times (homogenous suspension)
- Incubate at RT for 10 min
- Attract the beads (2 min) and discard the supernatant
- Leave the beads on the stand (!!!!) and add 300 µl ProNex Wash Buffer (Promega, NG2001)
- *Note: ensure that the beads are fully covered in wash buffer; if necessary, increase the volume for optimal washing efficiency*
- Incubate at RT for 1 min and discard the supernatant (carefully without loosing beads)
- Repeat washing once
- Discard the supernatant and let the beads air-dry at RT for 10 min (until cracking is visible)

- Remove the beads from the magnetic rack and resuspend them in 20 µL water (size selection control in 50 µL water)
- Incubate at RT for 5 min
- Attract the beads (2 min) and transfer the supernatant to a fresh tube

Library quality control and quantification

- Run 2 µL of the library, the size selection control, and ULR on a TapeStation or Fragment Analyzer and determine the concentration under the peak
- Calculate the ratio 150 bp and 75 bp band of the size-selected ULR Ladder (aiming for ~ 15)
  Note: *Ensure that no residual primers are left in the final library preparation. If primers are detected, repeat the ProNex size selection to remove them completely, as their presence can interfere with sequencing accuracy and data quality*
- Measure the sequencing library concentration twice with Qubit High Sensitivity D1000 Kit (Life Technologies) or similar technologies
- Calculate the final library quantification according to the method described by **Buchbender *et al.*, 2020**.
- *Note: Before submitting/ calculating the library, consult with your sequencing facility to confirm the required library concentration, volume, and quality.*
- Freeze the remaining library at - 80°C until sequencing

Oligos (IDT)

L3-App	/rApp/AGATCGGAAGAGCGGTTCAG/ddC/
RT_iCLIP2	GGATCCTGAACCGCT
L01_iCLIP2.0	/5Phos/NNNNATCACGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L02_iCLIP2.0	/5Phos/NNNNCGATGTNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L03_iCLIP2.0	/5Phos/NNNNTTAGGCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L04_iCLIP2.0	/5Phos/NNNNTGACCANNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L05_iCLIP2.0	/5Phos/NNNNACAGTGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L06_iCLIP2.0	/5Phos/NNNNGCCAATNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L07_iCLIP2.0	/5Phos/NNNNCAGATCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L08_iCLIP2.0	/5Phos/NNNNACTTGANNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L09_iCLIP2.0	/5Phos/NNNNGATCAGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L10_iCLIP2.0	/5Phos/NNNNTAGCTTNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L11_iCLIP2.0	/5Phos/NNNNATGAGCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L12_iCLIP2.0	/5Phos/NNNNCTTGTANNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L13_iCLIP2.0	/5Phos/NNNNAGTCAANNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L14_iCLIP2.0	/5Phos/NNNNAGTTCCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L15_iCLIP2.0	/5Phos/NNNNATGTCANNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L16_iCLIP2.0	/5Phos/NNNNCCGTCCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L17_iCLIP2.0	/5Phos/NNNNCAACTANNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L18_iCLIP2.0	/5Phos/NNNNGTCCGCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L19_iCLIP2.0	/5Phos/NNNNGTGAAANNNNNAGATCGGAAGAGCGTCGTG/3ddC/
L20_iCLIP2.0	/5Phos/NNNNCACCGGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/
P5_Solexa_S	ACACGACGCTCTTCCGATCT
P3_Solexa_S	CTGAACCGCTCTTCCGATCT
P5_Solexa	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
P3_Solexa	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Note: Buchbender *et al*., 2020 provides four additional barcode primers

Literature

Brachmann, A., Weinzierl, G., Kamper, J. & Kahmann, R. 2001. Identification of genes in the bW/bE regulatory cascade in Ustilago maydis. *Mol Microbiol,* 42**,** 1047-63.

Buchbender, A., Mutter, H., Sutandy, F. X. R., Kortel, N., Hanel, H., Busch, A., Ebersberger, S. & Konig, J. 2020. Improved library preparation with the new iCLIP2 protocol. *Methods,* 178**,** 33-48.