



# 🌐 An end-to-end workflow to study newly synthesized mRNA following rapid protein depletion in *Saccharomyces cerevisiae* V.2

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## ABSTRACT

In this protocol, we describe an end-to-end workflow for rapidly degrading a target protein using the AID system and quantifying newly synthesized mRNA using SLAM-seq in *Saccharomyces cerevisiae*. We describe methods for targeted protein degradation, 4-thiouracil (4tU) incorporation, rapid methanol fixation, RNA purification, RNA alkylation, and mRNA-seq library preparation. Although the individual methods described in this protocol are not novel per se, this workflow provides a complete resource for turnkey implementation of these methods, which will benefit others working with *S. cerevisiae*. In addition, this workflow is readily adaptable to other systems, including industrial, pathogenic, or other model fungi, which will benefit the larger research community.

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## GUIDELINES

Standard laboratory guidelines and practices should be followed when performing this protocol. To ensure accurate size selection, researchers should use careful and consistent pipetting when performing this protocol.

## MATERIALS

**Reagents:**

- Dimethyl sulfoxide, RNase-free (DMSO, ThermoFisher Scientific 327182500)
- 3-indoleacetic acid (IAA, Sigma I3750)
- 4-thiouracil (4tU, Sigma 440736)

## Note

*4tU is light sensitive and can crosslink upon exposure to light. Avoid exposure to light.*

- Dithiothreitol (DTT)
- DNA/RNA Shield (Zymo Research )
- DNase I, RNase-free (Zymo Research E1010)
- Iodoacetamide (Sigma I1149)
- Glycogen (20 mg/ml, Roche 10901393001)
- Sera-Mag SpeedBeads, magnetic carboxylate modified particles (Cytiva 65152105050250, ThermoFisher Scientific 09-981-123)

## Note

*Preparation of speedbeads and related buffers (DNA buffer and DNA binding buffer) is described in:*

## Protocol



NAME

**A method to prepare Sera-Mag SpeedBeads for purification and size selection of nucleic acids**

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PREVIEW

- Maxima H Minus Reverse Transcriptase (200 u/uL) and 5X first-strand buffer (ThermoFisher Scientific EP0752)
- 2X Ex Premier (DNA polymerase) master mix (Takara RR370A)
- dNTP solution set, dATP, dCTP, dGTP, and dTTP (NEB N0446S)
- RNase H (NEB M0297L)
- IRA\_UMI\_24dTVN oligo (PAGE purification, IDT)
- IFA-isoTSO oligo (standard purification, IDT)

- Working stocks (5 µM) of i5 (i5-D501 through i5-D508) and i7 (i7-D701 through i7-D512) oligo mixes (standard purification, IDT)

### Note

*Oligo sequences and methods for oligo preparation are described in (see also Foley et al., 2019, Glenn et al., 2019, and Veeranagouda et al., 2019):*

### Protocol



NAME

**High-throughput sequencing (HTS) oligos and methods to prepare oligos for HTS applications**

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[PREVIEW](#)

- 100% methanol
- 100% ethanol
- Nuclease-free or DEPC-treated water
- MilliQ water

#### Solutions:

- 10 mM Tris-HCl (pH 8.0)
- 0.5 M sodium phosphate buffer (pH 8.0, prepared with nuclease-free or DEPC-treated water)
- 3 M sodium acetate (pH 5.2, prepared with nuclease-free or DEPC-treated water)
- 1 M DTT
- 100 mM DTT
- 20 mM DTT
- 80% ethanol (freshly prepared with nuclease-free or DEPC-treated water)

#### Buffers:

- Speedbead DNA buffer
- Speedbead DNA binding buffer

#### Consumables:

- 50 ml conical tubes (sterile)

- 1.5 ml microcentrifuge tubes (nuclease-free)
- 0.2 ml PCR tubes or strips (low-bind, nuclease-free)
- Quick-RNA Fungal/Bacterial Miniprep kit (Zymo Research R2014)

#### Note

*Other RNA extraction/purification methods can be substituted. Alternative methods should be modified to maintain reducing conditions.*

#### Equipment:

- Mini-Beadbeater-24 (Biospec Products)

#### Note

*Other bead beaters or cell homogenizers can be substituted. Processing conditions should be adjusted accordingly.*

- Thermomixer (e.g., ThermoMixer F1.5, Eppendorf) or dryblock
- Magnetic separation rack for 0.2 ml PCR tubes (e.g., NEBNext Magnetic Separation Rack, NEB)

#### Note

See Sergi Lab Supplies for economical and effective magnetic separation racks.

#### SAFETY WARNINGS



Standard safe laboratory practices and procedures and institution-specific waste management programs should be followed when performing this protocol.

## Yeast growth

- 1 Streak appropriate *S. cerevisiae* strain on YPD agar. Incubate culture at 30°C for 2-3 days.

**Note**

*YPD agar and YPD are prepared and supplemented with adenine following standard procedures. Other media can be used.*

- 2 Transfer an individual colony to 5 ml of YPD. Incubate culture at 30°C with shaking at 220 rpm overnight.
- 3 Dilute overnight culture to an OD<sub>600</sub> of 0.2 in 40 ml of YPD. Incubate culture at 30°C with shaking at 220 rpm.
- 4 As the 40 ml culture approaches an OD<sub>600</sub> of 0.3-0.4, be prepared to proceed with IAA treatment, 4tU labeling, sampling, and rapid fixation.

### IAA treatment, 4tU labeling, sampling, and rapid fixation

- 5 In preparation for IAA treatment, 4tU labeling, sampling, and rapid fixation, cool centrifuge to 4°C and prepare 50 ml conical tubes, 2.0 ml microcentrifuge tubes, and 1.5 ml microcentrifuge tubes.

**Note**

*IAA treatment and 4tU treatment are based on Donczew et al., 2020 and Donczew and Hahn, 2021. Sampling and rapid fixation methods are based on Barrass et al., 2015, Aslanzadeh et al., 2018, and Barrass and Beggs, 2019 (see also Koš and Tollervey, 2010 and Kara et al., 2021). Samples should be protected from light during Steps 12-22 when possible (e.g., work under red light or low light and keep samples covered with foil).*

- 6 Add 5 ml of 100% methanol to a 50 ml conical tube for each sample to be collected. Prechill prepared and labeled tubes on dry ice. Two 50 ml conical tubes are needed for each 40 ml culture prepared in Step 4.

- 7 When the 40 ml culture reaches an OD<sub>600</sub> of 0.4-0.5, split the culture into three 10 ml aliquots in 50 ml conical tubes and return them to the incubator.

Each 10 ml culture represents one of three treatments:

| A         | B             | C             |
|-----------|---------------|---------------|
| Treatment | IAA treatment | 4tU treatment |
| A         | DMSO          | 4tU           |
| B         | IAA           | 4tU           |
| C         | DMSO          | DMSO          |

**Note**

*DMSO is the carrier for both IAA and 4tU. Treatment C is a control treatment and may not be required for all experiments. For example, once background T>C conversion rates have been established for an experimental system Treatment C may not be required.*

- 8 Equilibrate cultures at 30°C for 5 min with shaking at 220 rpm.
- 9 During equilibration (or just prior to splitting the culture), prepare a 0.2 M IAA (3-indoleacetic acid) solution in DMSO. IAA is freshly prepared. To prepare a 0.2 M IAA solution, add 220 mg of IAA to 6 ml of DMSO and vortex until dissolved. Keep the IAA solution at room temperature covered with foil.
- 10 **CRITICAL STEP:** Add 50 µl of 0.2 M IAA solution or DMSO to the appropriate 10 ml culture. **Mix the culture vigorously and return it to the incubator.** The final concentration of IAA is 1 mM.
- 11 Incubate cultures at 30°C for 25 min with shaking at 220 rpm.

**Note**

*Cultures are generally incubated for 25 min for protein degradation prior to adding 4tU. The total time for protein degradation is ~30 min. The time can be adjusted based on the experiment.*

- 12 During IAA treatment, prepare a 2 M 4tU (4-thiouracil) solution in DMSO. 4tU is freshly prepared. To prepare a 2 M 4tU solution, add 260 mg of 4tU to 1 ml of DMSO and vortex until dissolved. Keep the 4tU solution at room temperature covered with foil.
- 13 **CRITICAL STEP:** Add 25  $\mu$ l of 2 M 4tU solution or DMSO to the appropriate 10 ml culture. **4tU will precipitate when added to the culture; mix the culture vigorously and return it to the incubator.** The final concentration of 4tU is 5 mM. It is essential that cultures are mixed vigorously and uniformly to ensure 4tU is dissolved and homogeneously distributed.
- 14 Incubate cultures at 30°C for 4 min with shaking at 220 rpm.

**Note**

*Cultures are generally incubated for 4 min for 4tU incorporation. The time can be adjusted based on the experiment.*

- 15 **CRITICAL STEP: Immediately after 4tU treatment, decant each 10 ml culture directly into 5 ml of 100% methanol in a 50 ml conical tube prechilled on dry ice (prepared in Step 6).** Loosely cap the slurry and gently mix by swirling to ensure homogenization. Keep the fixed sample on dry ice. The final concentration of methanol is approximately 33%.

**Note**

**PAUSE POINT:** Samples in methanol can be stored at -80°C. Thaw samples stored at -80°C at room temperature until frozen media has melted (approximately 5 min) before proceeding.

- 16 Gently mix the fixed sample and transfer 250  $\mu$ l to a 1.5 ml microcentrifuge tube. Store the 250  $\mu$ l aliquot at 4°C for cell counting. Estimate cell number using OD<sub>600</sub> or count cells using a Neubauer chamber.

**Note**

The estimated cell number will be used to normalize samples for western blotting. It is generally sufficient to measure the OD for one treatment (e.g., Treatment A) for a given strain or replicate – the results for all treatments should be identical.

- 17 Gently mix the fixed sample and transfer 1.5 ml to a labeled 2.0 ml microcentrifuge tube for western blotting. Centrifuge the sample at max speed for 1 min to pellet cells, discard the supernatant, and freeze cell pellets at -20°C.

**Note**

**PAUSE POINT:** Store cell pellets at -20°C (up to 2 weeks) or -80°C (long-term storage). The volume of culture collected is 1 ml. It is helpful to record the OD value (Step 16) on the 2.0 ml microcentrifuge tube for future reference.

- 18 Centrifuge the remaining slurry (approximately 13 ml) at 3,000 g and 4°C for 10 min to pellet cells.
- 19 Carefully decant supernatant and invert the 50 ml conical tube on a paper towel to remove residual supernatant
- 20 Thoroughly resuspend cells in an appropriate volume of DNA/RNA Shield by pipetting. Adjust samples to a uniform cell number using the following formula to calculate the appropriate volume of buffer for each sample:

$$\text{Volume of buffer needed} = (\text{final OD}_{600} \text{ of sample}/0.70)*400.$$

- 21 Ensure cells are thoroughly resuspended and transfer 400 µl of the cell suspension to a labeled 1.5 ml microcentrifuge tube.

22 **PAUSE POINT:** Flash freeze samples on dry ice or in liquid nitrogen and store at -80°C until proceeding.

## RNA purification and DNase I treatment

23 Supplement the following buffers and reagents with DTT (100 mM DTT working stock):

| A                   | B                       | C                   | D               |
|---------------------|-------------------------|---------------------|-----------------|
| Reagent             | Reagent vol. per sample | DTT vol. per sample | Final DTT conc. |
| RNA Lysis Buffer    | 400 µl                  | 0.8 µl              | 0.2 mM          |
| RNA Wash Buffer     | 1.5 ml                  | 1.5 µl              | 0.1 mM          |
| RNA Prep Buffer     | 400 µl                  | 0.4 µl              | 0.1 mM          |
| 100% ethanol        | 550 µl                  | 0.55 µl             | 0.1 mM          |
| Nuclease-free water | 50 µl                   | 0.5 µl              | 1 mM            |

### Note

*RNA purification and DNase I treatment is performed using a Quick-RNA Fungal/Bacterial Miniprep kit (see Materials). Methods are based on manufacturer's recommendations modified to maintain reducing conditions. DTT is used during RNA purification to maintain reducing conditions. Prewarm the required volume of nuclease-free water (supplemented with DTT) (see Step 41). Perform all centrifugation steps at 16,000 g and room temperature for 30 s (unless specified). Samples should be protected from light during Steps 23-42 when possible (e.g., work under red light or low light and keep samples covered with foil).*

24 Thaw samples stored in DNA/RNA Shield at -80°C at room temperature.

25 Combine 400 µl of RNA Lysis Buffer (supplemented with DTT) and 400 µl of cells suspended in DNA/RNA Shield adjusted as described in Step 20.

- 26 Transfer 800 µl of cells suspended in 1:1 DNA/RNA Shield:RNA Lysis Buffer (supplemented with DTT) to a ZR Bashingbead Lysis Tube.
- 27 Process samples in a Mini-Beadbeater-24 at 3800 rpm for 45 s and immediately incubate on ice for 2 min.
- 28 Repeat Step 27 once.
- 29 While samples are incubating on ice, prepare the following reaction mixture in a 1.5 ml microcentrifuge tube on ice:

**DNase I reaction mixture**

| A                               | B                     | C                    |
|---------------------------------|-----------------------|----------------------|
| Reagent                         | 1X reaction vol. (µl) | Master mix vol. (µl) |
| DNA Digestion Buffer            | 35                    |                      |
| DNase I, reconstituted (1 u/µl) | 5                     |                      |
| Total                           | 40                    |                      |

**Note**

*Do not supplement DNA Digestion Buffer with DTT.*

- 30 Centrifuge samples at 16,000 g for 1 min to pellet debris.
- 31 **CRITICAL STEP:** Transfer 550 µl of the cleared supernatant to a Zymo-spin IIICG Column in a collection tube and centrifuge at 16,000 g for 30 s. **Save the flow through.**

- 32 Add an equal volume (550 µl) of 100% ethanol (supplemented with DTT) to the flow through. Thoroughly mix samples by pipetting
- 33 Transfer 700 µl of the mixture to a Zymo-spin IICR Column in a collection tube and centrifuge at 16,000 g for 30 s. Discard flow through.
- 34 Repeat Step 34 with the remaining mixture volume.
- 35 Add 400 µl of RNA Wash Buffer (supplemented with DTT) to the column and centrifuge at 16,000 g for 30 s. Discard flow through.
- 36 Gently mix DNase I reaction mixture (prepared in Step 29) by inversion and add 40 µl directly onto column matrix and incubate at room temperature for 15 min. DNase I is easily denatured. Do not vortex reaction mixture.
- 37 Add 400 µl of RNA Prep Buffer (supplemented with DTT) to the column and centrifuge at 16,000 g for 30 s. Discard flow through.
- 38 Add 700 µl of RNA Wash Buffer (supplemented with DTT) to the column and centrifuge at 16,000 g for 30 s. Discard flow through.
- 39 Add 400 µl of RNA Wash Buffer (supplemented with DTT) to the column and centrifuge at 16,000 g for 1 min to ensure complete removal of buffer.

- 40 Carefully transfer the column to a labeled nuclease-free 1.5 ml microcentrifuge tube.
- 41 Add 50  $\mu$ l of nuclease-free water (supplemented with DTT) prewarmed to 50°C directly to column matrix, incubate at room temperature for 2 min, and centrifuge at 16,000 g for 30 s.
- 42 **PAUSE POINT:** Store purified RNA samples at -20°C (up to eight weeks) or -80°C until proceeding.

## RNA alkylation and ethanol precipitation

- 43 Thaw RNA samples on ice. Quantify RNA using a Nanodrop.

### Note

*RNA alkylation (iodoacetamide treatment) method is based on Herzog et al., 2017 (see also Alalam et al., 2022). Alternatively, the SLAMseq Kinetics kit – Anabolic Kinetics module (Lexogen) can be used for alkylation and ethanol precipitation following the manufacturer's recommendations. Iodoacetamide is referred to as IAA in Herzog et al., 2017; IAA refers to 3-indoleacetic acid in this protocol. Samples should be protected from light during Steps 43-49 when possible (e.g., work under red light or low light and keep samples covered with foil).*

- 44 Add 5  $\mu$ g of total RNA to a 1.5 ml microcentrifuge tube on ice. Adjust the volume of each sample to 20  $\mu$ l with nuclease-free water.
- 45 Prepare a 100 mM iodoacetamide solution in DMSO. Iodoacetamide is freshly prepared. To prepare a 100 mM iodoacetamide solution, add 9.25 mg of iodoacetamide to 500  $\mu$ l of DMSO (1 mg of iodoacetamide to

54 µl of DMSO). Keep the iodoacetamide solution at room temperature covered with foil. Do not store unused iodoacetamide.

- 46** Prepare the following reaction mixture in a 1.5 ml microcentrifuge tube and mix well by pipetting:

**Alkylation reaction mixture**

| A                                      | B                     | C                    |
|--|-----------------------|----------------------|
| Reagent                                | 1X reaction vol. (µl) | Master mix vol. (µl) |
| 100 mM iodoacetamide                   | 5                     |                      |
| 0.5 M sodium phosphate buffer (pH 8.0) | 5                     |                      |
| DMSO                                   | 20                    |                      |
| Total                                  | 30                    |                      |

**Note**

*The sodium phosphate may form aggregates in the reaction mixture. The presence of aggregates will not interfere with the reaction. Prepare an additional 10% volume of master mix to avoid transferring the aggregates.*

- 47** Add 30 µl of reaction mixture to the 1.5 ml microcentrifuge tube containing 5 µg of total RNA in 20 µl nuclease-free water (prepared in Step 44).

- 48** Gently mix reaction and incubate in a thermomixer at 900 rpm and 50°C for 15 min in the dark.

- 49** Add 1 µl of 1 M DTT to stop the reaction. Briefly vortex to mix. Exposure to light is acceptable following the addition of 1 M DTT.

- 50** Add 1 µl of glycogen (20 mg/ml) and 5 µl of 3 M sodium acetate (pH 5.2) to each sample. Briefly vortex to mix.

- 51 Add 125 µl of 100% ethanol to each sample. Briefly vortex to mix and incubate at -80°C for 30 min.
- 52 Centrifuge samples for 30 min at 21,000 g and 4°C to pellet precipitated RNA.
- 53 Carefully decant the supernatant. Take care not to dislodge pellet.
- 54 Add 1 ml of 80% ethanol to each sample. Centrifuge samples for 10 min at 21,000 g and 4°C to wash pellet.
- 55 Carefully decant the supernatant. Take care not to dislodge pellet. Carefully inspect samples and remove remaining ethanol with a 20 µl pipet.
- 56 Air dry samples for 5 min with lid open. Do not over dry samples.
- 57 Resuspend RNA in 30 µl of nuclease-free water.
- 58 **PAUSE POINT:** Store alkylated RNA samples at -80°C or proceed directly with library preparation.

## 3' mRNA sequencing library preparation

### 59 RNA fragmentation and cDNA synthesis.

#### Note

*3' mRNA sequencing library preparation is based on Foley et al., 2019 and Veeranagouda et al., 2019. Prewarm required volume of 10 mM Tris-HCl (pH 8.0) (see Steps 60.4 and 61.10).*

- 59.1 Thaw iodoacetamide-treated RNA samples on ice. Quantify RNA using an RNA-specific fluorometry method (i.e., Qubit RNA BR or HS assay kit).
- 59.2 Add 200 ng of RNA (total RNA) to a 0.2 ml PCR tube or strip on ice. Adjust the volume of each sample to 5 µl with nuclease-free water.
- 59.3 Prepare the following reaction mixture in a 0.2 ml PCR tube (or 1.5 ml microcentrifuge tube) on ice and mix well by pipetting:

#### Fragmentation reaction mixture

|                                 | A   | B                     | C                    |
|---------------------------------|-----|-----------------------|----------------------|
| Reagent                         |     | 1X reaction vol. (µl) | Master mix vol. (µl) |
| 5X reverse transcription buffer | 2   |                       |                      |
| 20 mM dNTP mix                  | 0.5 |                       |                      |
| 20 µM IRA_UMI_24dTVN oligo      | 0.5 |                       |                      |
| Total                           | 3   |                       |                      |

- 59.4 Prepare the following reaction mixture in a 0.2 ml PCR tube (or 1.5 ml microcentrifuge tube) on ice and mix well by pipetting:

#### Reverse transcription (RT) reaction mixture

| A                                | B                     | C                    |
|----------------------------------|-----------------------|----------------------|
| Reagent                          | 1X reaction vol. (μl) | Master mix vol. (μl) |
| 20 mM DTT                        | 1                     |                      |
| 20 μM IFA-isoTSO oligo           | 0.5                   |                      |
| Reverse transcriptase (200 u/μl) | 0.5                   |                      |
| Total                            | 2                     |                      |

**59.5** Bring the RNA and fragmentation reaction mixture to room temperature for 2-5 minutes and add 3 μl of the fragmentation reaction mixture to each sample. Mix well by pipetting.

**59.6** **CRITICAL STEP: Incubate samples at 94°C for exactly 4 min in a thermocycler with heated lid to fragment the RNA.** Cool samples to 42°C in a thermocycler. Centrifuge briefly at room temperature to collect samples. Return samples to a thermocycler at 42°C. Fragmentation time can be optimized to generate libraries of different insert sizes.

**59.7** Bring the RT reaction mixture to room temperature for 2-5 minutes prepared in Step 59.4. Add 2 μl of the RT reaction mixture to each sample and mix well by pipetting. Centrifuge briefly at room temperature to collect samples. Return samples to thermocycler at 42°C.

**59.8** Heat samples to 42°C for 60 min followed by 70°C for 10 min in a thermocycler with heated lid. Cool samples to 37°C in a thermocycler. Proceed immediately to RNA removal (Step 60).

## 60 RNA removal and single-stranded DNA purification.

**60.1** Add 1 μl of RNase H to each sample and mix well by pipetting. Centrifuge briefly at room temperature to collect samples.

60.2 Heat samples to 37°C for 15 min in a thermocycler with heated lid. Proceed immediately to speedbead purification.

60.3 Purify the single-stranded DNA (ssDNA). Add 39 µl of DNA buffer and 1 volume (50 µl) of speedbeads to the 0.2 ml PCR tube containing the ssDNA and proceed as described in the general method for speedbead purification (Step 65).

**Note**

*Ensure speedbeads are equilibrated to room temperature and thoroughly mixed. DNA buffer and speedbeads can be mixed prior to use (here and in Step 61.4). If using commercial beads, it may be necessary to supplement beads with 0.05% Tween 20.*

60.4 Elute the ssDNA in 21 µl of 10 mM Tris-HCl (pH 8.0) prewarmed to 50°C.

60.5 Transfer 20 µl of the cleared supernatant to a fresh PCR tube.

60.6 **PAUSE POINT:** Store purified ssDNA at -20°C or proceed directly with barcoding and enrichment PCR and library purification.

## 61 Barcoding and enrichment PCR and library purification.

61.1 Transfer 10 µl of purified ssDNA to a 0.2 ml PCR tube on ice.

- 61.2** Add 2.5 µl of an appropriate 5 µM i5/i7 primer mix to each sample on ice.
- 61.3** Add 12.5 µl of 2X Ex Premier master mix to each sample on ice. Mix thoroughly by pipetting.
- 61.4** Barcode and enrich the library using PCR. Perform initial denaturation at 98°C for 45 s, followed by 12-18 cycles of amplification (98°C for 10 s, 60°C for 15 s, and 68°C for 15 s), and bring the reactions to 20°C.

**Note**

*Use 12-14 amplification cycles if starting with ≥500 ng of total RNA, 16 amplification cycles if starting with 200 ng of total RNA, or 18 amplification cycles if starting with 100 ng of total RNA.*

- 61.5** Purify the library. Add 25 µl of DNA buffer and 0.8 volumes (40 µl) of speedbeads to the 0.2 ml PCR tube containing the amplified library.
- 61.6** Mix well by pipetting up and down a minimum of 10 times. Alternatively, vortex samples on setting 4 for 3-5 seconds. If samples require centrifugation after mixing, stop the centrifuge before beads settle.
- 61.7** Incubate samples at room temperature for 5 min.

- 61.8 Place samples on magnetic stand for 2-5 min (or until solution is completely clear) to collect beads. Carefully remove and discard cleared supernatant without disturbing the bead pellet.
- 61.9 Remove samples from the magnetic stand. Add 30 µl of DNA buffer and mix well by pipetting or vortexing. Ensure beads are fully resuspend and incubate samples at room temperature for 2 min.
- 61.10 Add 0.8 volumes (24 µl) of DNA binding buffer to the 0.2 ml PCR tube containing the library and bead suspension. Proceed as described in the general method for speedbead purification (Step 65).
- 61.11 Elute the library in 21 µl of 10 mM Tris-HCl (pH 8.0) prewarmed to 50°C.
- 61.12 Transfer 20 µl of the cleared supernatant to a fresh PCR tube.
- 61.13 **PAUSE POINT:** Store the purified sequencing library at -20°C.

### General method for speedbead purification

- 62 Equilibrate speedbeads to room temperature and mix thoroughly.
- 63 Carefully add indicated amount of speedbeads to the sample.

- 64 Mix well by pipetting up and down a minimum of 10 times. Alternatively, vortex samples on setting 4 for 3-5 seconds. If samples require centrifugation after mixing, stop the centrifuge before beads settle.
- 65 Incubate samples at room temperature for 5 min. Prolonged incubation or incubation at low temperature will increase binding of small nucleic acids (e.g., adapter dimers).
- 66 Place samples on magnetic stand for 2-5 min (or until solution is completely clear) to collect beads. Keep samples on magnetic stand for Steps 66-70.
- 67 Carefully remove and discard cleared supernatant without disturbing the bead pellet.
- 68 Add 200 µl of 80% ethanol (freshly prepared with MilliQ water) and incubate for 30 s. Carefully remove ethanol and discard without disturbing the bead pellet.
- 69 Repeat Step 68 once. Carefully inspect samples and remove remaining ethanol using a 10 µl pipet.
- 70 Air dry samples for 2 min with lid open. Do not over-dry samples. Speedbeads (and other SPRI products) will clump and can not be fully resuspended during elution if samples are over-dried.

- 71 Remove samples from the magnetic stand. Add the indicated volume of appropriate solution for elution and mix well by pipetting or vortexing (see Step 64).
- 72 Incubate mixture at room temperature for 2-5 min.
- 73 Place samples on a magnetic stand for 2 min (or until solution is completely clear) to collect beads.
- 74 Transfer cleared supernatant (volume of solution added for elution less 1  $\mu$ l) to a fresh PCR tube.