TriLink BIOTECHNOLOGIES
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CleanTag® Small RNA Library Preparation Kit

Product Insert

Cat. No. L-3206
24 Reactions

CleanTag® Small RNA Library Prep Kit contains chemically modified adapters and reagents to convert small RNA having 5' phosphate and 3' hydroxyl groups to corresponding libraries for next-generation sequencing. Multiplexing is facilitated using indexed PCR primers selected by the user that are compatible with either Illumina® or Ion Torrent™ sequencing systems.

CleanTag chemically modified adapters greatly suppress adapter dimer formation during adapter ligations and are optimized for total RNA input from 1-1,000 nanograms. Inputs as low as 10 picograms have been successfully sequenced but results with such low input amounts may vary depending on sample quality and application. For more details on this technology see Reference 1.

Please note, before you begin it is important to plan your sequencing experiment. Refer to sequencing platform manufacturer's instructions as a guide for barcoding with proper index primers.

Kit Components

Component Label	Details
CleanTag 3' Adapter	PO DNA, 5' (rApp) TGG AAT TCT CGG GTG CCA AGG (ddC) 3'; CleanTag Modified
CleanTag 5' Adapter	PO RNA, 5' GUU CAG AGU UCU ACA GUC CGA CGA UC 3'; CleanTag Modified
Enzyme 1	T4 RNA Ligase 2, truncated KQ
Enzyme 2	T4 RNA Ligase 1 (ssRNA Ligase)
Buffer 1	3' Adapter Ligation Buffer
Buffer 2	5' Adapter Ligation Buffer
RNase Inhibitor	Recombinant murine RNase Inhibitor
dNTP Mix	10 mM each dATP, dCTP, dGTP, dTTP
Reverse Transcriptase	ProtoScript® II Reverse Transcriptase (M-MuLV, RNase H minus)
RT Buffer	Reverse Transcription Buffer
DTT (100 mM)	Dithiothreitol
High Fidelity PCR Master Mix	Q5® High Fidelity 2X Master Mix

Store kit components at-20°C. Do not expose enzymes to prolonged temperatures above-20°C.
Use only certified RNase-free reagents and consumables with proper RNase-free technique.
Pulse spin centrifuge vials to collect liquid before first use due to small volumes.

Customer Supplied Materials and Equipment

Recommended Cleaning Supplies:

- 10% bleach
- RNase-Zap™ (Thermo cat no AM9780)
- 70% isopropanol wipes (Fisher cat no 06-665-24)

Library Preparation Materials and Equipment:

- Total RNA or purified small RNA
- Nuclease-free water
- Nuclease-free pipet tips and tubes
- Microcentrifuge tubes (at least 1.5 mL)
- Thin walled PCR tubes (0.2 ml strip or individual tubes)
- Ice or cold-block
- Microcentrifuge
- PCR Thermocycler with heated lid

RT, PCR Multiplexing Primers

NOTE: CleanTag Small RNA Library Preparation Kit does not include the RT Primer or PCR Primer sets, however these primers are necessary to complete library preparation and can be purchased online from TriLink. Choose the appropriate primers for your NGS system from the catalog items below for your experiment.

Illumina Compatible Index Primer Sets

- L-3204: Set 1 [RT primer, Forward PCR Primer, Reverse PCR Primers (Indexes 1-12)]
- L-3205: Set 2 [RT primer, Forward PCR Primer, Reverse PCR Primers (Indexes 13-24)]
- L-3207: Set 3 [RT primer, Forward PCR Primer, Reverse PCR Primers (Indexes 25-36)]
- L-3208: Set 4 [RT primer, Forward PCR Primer, Reverse PCR Primers (Indexes 37-48)]

Ion Torrent Compatible Barcode Convert Primer Sets

- L-3210: Set 1 [RT primer, Forward PCR Primers (Barcodes 1-12), Reverse PCR Primers]
- L-3211: Set 2 [RT primer, Forward PCR Primers (Barcodes 13-24), Reverse PCR Primers]

Analytical Materials and Equipment:

The following items (or similar) may be used for RNA or library analysis (user selected)

- 2100 Bioanalyzer (Agilent)
- Agilent 2100 Small RNA Kit (Agilent cat. no. 5067-1548)
- Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)
- Electrophoresis equipment and consumables (4% Agarose or 6% PAGE) with SYBR gold stain or equivalent

Library Purification Materials and Equipment:

The following purification materials (or similar) may be used. Library purification method is determined by user.

- AMPure® XP Beads (Beckman Coulter, Inc.)
  - 70% Ethanol
  - Nuclease-free Water, TRIS-Acetate (10 mM pH 8.0), or TE Buffer (10 mM Tris-Acetate pH 8.0, 1 mM EDTA)
  - Magnetic Separation Stand
  - 2100 Bioanalyzer (Agilent®)
  - Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)
- Electrophoresis equipment and consumables
  - 4% Agarose EX E-gel (Thermo Fisher cat. no. G401004) or 6% PAGE gel
  - SYBR gold stain or equivalent
  - Invitrogen 50 bp DNA Ladder
  - ZymoClean Gel DNA Recovery Kit (Zymo Research cat. no. D4001T)
- Pippin Prep (Sage Science) and 3% Agarose Dye Free Gel (Sage Science #CDF 3010)
- PureLink™ PCR Micro Kit (Thermo Fisher cat. no. K310050)

Small RNA Extraction

The success of NGS experiments involving small RNA library can be strongly influenced by the reproducibility and recovery of small RNA species from your samples[2-4]. Bioanalyzer analysis of extracted small RNA is highly recommended prior to library preparation.

RNA Input Recommendations

1 ng to 1 µg of Total RNA

Note See recommended adapter dilutions in STEP 1, Table 1 and PCR Cycling Conditions in STEP 4, Table 10 for different input levels.

Always clean work area and pipets before working with RNA.

- Use 10% bleach, water, RNase-Zap, and isopropanol to clean bench top and pipets.
- Only use nuclease-free consumables to prevent degradation of RNA material.

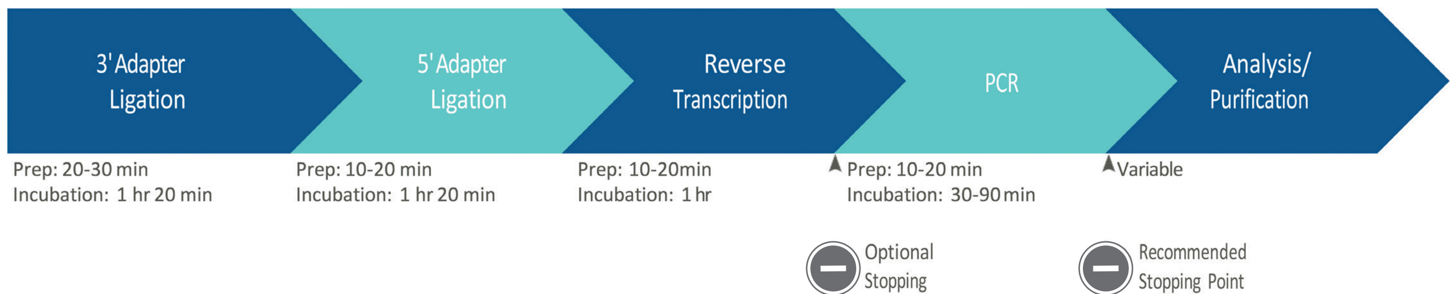
Proper mixing of all reagents within each reaction is crucial for optimal library preparation.

- Pipet up and down gently 2-3 times to mix each new component before withdrawing a solution from stock vial.
- Pipet up and down gently 2-3 times to mix newest reagent with existing mixture in each reaction tube.
- Ensure all liquid has been dispensed from pipet tip before proceeding.
- Mix full reactions before incubation by pipetting up and down 5-10 times.
- Avoid bubbles when pipetting.
- Use clean tips between samples to prevent cross contamination.
- Protocol includes preparing master mixes for the four enzymatic steps of library prep. Volumes are given per reaction and for a 3 reaction master mix. To calculate volumes for larger or smaller number of reactions, use the following formula:

  (No. of Rxn) x (Single Rxn Volume) x (1.1) = total volume required per master mix component.

CleanTag Small RNA Library Preparation Workflow

Note: It is recommended to complete the protocol through the PCR step. If necessary, an optional stop point is available after the RT step.



References

1. Shore S, Henderson JM, Lebedev A, Salcedo MP, Zon G, McCaffrey AP, Paul N, Hogrefe RI. Small RNA Library Preparation Method for Next-Generation Sequencing Using Chemical Modifications to Prevent Adapter Dimer Formation. PLoS One. 2016 Nov 22;11(11):e0167009. eCollection 2016.
2. Sellin Jeffries MK, Kiss AJ, Smith AW, Oris JT. A comparison of commercially-available automated and manual extraction kits for the isolation of total RNA from small tissue samples. BMC Biotechnol. 2014 Nov 14;14:94.
3. Wang WX, Wilfred BR, Baldwin DA, Isett RB, Ren N, Stromberg A, Nelson PT. Focus on RNA isolation: obtaining RNA for microRNA (miRNA) expression profiling analyses of neural tissue. Biochim Biophys Acta. 2008 Nov;1779(11):749-57. Epub 2008 Feb 13.
4. McAlexander MA, Phillips MJ, Witwer KW. Comparison of Methods for miRNA Extraction from Plasma and Quantitative Recovery of RNA from Cerebrospinal Fluid. Front Genet. 2013 May 16;4:83. eCollection 2013.

Protocols

STEP 1: 3' Adapter Ligation to RNA Template

1. Thaw CleanTag 3' Adapter and Buffer 1 and place on ice. Keep Enzyme 1 and RNase Inhibitor on ice.

Note: For RNA inputs <1000 ng, dilute CleanTag 3' Adapter as recommended in Table 1.

Table 1: Low RNA Input Adapter Dilution

Total RNA Template Input	Adapter Dilution
1,000 ng	1X
100 ng	1:2
10 ng	1:4
1 ng	1:12

Note: Some components and mixtures may be viscous, pipet slowly.
Do not vortex enzymes.

2. Prepare a master mix containing all components in the order shown in Table 2 in a 1.5 mL tube. Keep on ice.

Table 2: 3' Adapter Ligation Master Mix Set Up

Component	Volume per Reaction	Volume for 3 Reactions with 10% excess
CleanTag 3' Adapter	1.0 µL	3.3 µL
RNase Inhibitor	1.0 µL	3.3 µL
Enzyme 1	1.0 µL	3.3 µL
Buffer 1	5.0 µL	16.5 µL
Total Volume (µL)	8 µL	26.4 µL

Table 3: 3' Adapter Reaction Volume

Component	Volume per Reaction
Master Mix	8.0 µL
RNA Template[1]	≤ 2.0 µL
Nuclease-free water	as needed to total 10 µL
Total Volume (µL)	10 µL

[1]Up to 10 µL RNA template is tolerated in reaction. Take entire final reaction volume forward.

3. Mix gently to protect enzyme by pipetting up and down. Do not vortex. Pulse spin to collect liquid if necessary.
4. Heat RNA template at 70°C for 2 minutes and place immediately on ice.
5. Aliquot 8 µL of master mix into thin walled PCR tubes.
6. Add up to 2 µL RNA template to appropriate tube. Add nuclease-free water as needed to bring volume to 10 µL per tube as shown in Table 3. Keep on ice.
7. Using a new pipet tip for each tube, set pipet volume to 8 µL and gently mix volume 5-10 times by pipetting up and down slowly (CAUTION: mixture is still viscous). Cap tubes and pulse spin to collect liquid.
8. Place the tubes into a thermal cycler with a heated lid and perform the incubation conditions:
   28°C for 1 hour
   65°C for 20 min
9. Proceed immediately to STEP 2.

STEP 2: 5’ Adapter Ligation to 3’ Tagged RNA Template

1. Thaw CleanTag 3’ Adapter and Buffer 1 and place on ice. Keep Enzyme 1 and RNase Inhibitor on ice.

Note: For RNA inputs <1000 ng, dilute CleanTag 5’ Adapter following the same technique as the CleanTag 3’ Adapter shown in Table 1.

Note: Some components and mixtures may be viscous, pipet slowly. Do not vortex enzymes.

2. While on ice, prepare master mix with components from Table 4, in the order of addition shown, in a 1.5 mL tube.

Table 4: 5’ Ligation Adapter Master Mix Set Up

Component	Volume per Reaction	Volume for 3 Reactions with 10% excess
Nuclease-free water	4.0 µL	13.2 µL
Buffer 2	1.0 µL	3.3 µL
RNase Inhibitor	1.0 µL	3.3 µL
Enzyme 2	2.0 µL	6.6 µL
Total Volume (µL)	8 µL	26.4 µL

Table 5: 5’ Adapter Reaction Volume

Component	Volume per Reaction
3' Tagged RNA Template from STEP 1	10.0 µL
Master Mix	8.0 µL
CleanTag 5' Adapter	2.0 µL
Total Volume (µL)	20 µL

3. Add 8.0 µL of master mix to each 3’ Tagged RNA Template from STEP 1. Keep on ice.
4. Heat CleanTag 5’ Adapter at 70°C for 2 minutes and place immediately on ice.
5. Add 2 µL CleanTag 5’ Adapter to each reaction tube to meet final volume shown in Table 5, mix by pipetting up and down, pulse spin to collect liquid.
6. While on ice, with a new pipet tip, set volume to 18 µL and mix volume 5-10 times by pipetting up and down gently. Mix each reaction with a new pipet tip. Cap tubes and pulse spin to collect liquid.
7. Place the tubes into a thermal cycler with a heated lid and perform the incubation conditions:
   28°C for 1 hour
   65°C for 20 min
8. Place Tagged RNA library on ice and proceed immediately to STEP 3.

STEP 3: Reverse Transcription (RT) Reaction of Tagged RNA Library

RT Primer is included in each separate indexed Primer Set (for Illumina cat. no. L-3204, L-3205, L-3207, or L-3208; for Ion Torrent cat no. L-3210, or L-3211)

1. Thaw RT Primer, RT Buffer, dNTPs, and DTT and place on ice. Keep RT Enzyme on ice and do not vortex.
2. Add 2.0 µL of RT Primer to Tagged Library as shown in Table 6. Mix by pipetting up and down, pulse spin to collect liquid.
3. Heat tubes at 70°C for 2 min. Immediately place on ice.
4. Prepare master mix with components in Table 6. Add components in order shown into a 1.5 mL tube.

Table 6: RT Master Mix Set Up

Component	Volume per Reaction	Volume for 3 Reactions with 10% excess
Nuclease-free water	1.92 µL	6.34 µL
RT Buffer (5X)	5.76 µL	19 µL
dNTPs (10 mM)	1.44 µL	4.75 µL
DTT (100 mM)	2.88 µL	9.5 µL
RNase Inhibitor	1.00 µL	3.3 µL
RT Enzyme (200 U/µL)	1.00 µL	3.3 µL
Total Volume (µL)	14.0 µL	46.19 µL

Table 7: RT Reaction Volume

Component	Volume per Reaction
Tagged Library from STEP 2[1]	20.0 µL
RT Primer (5 µM)[2]	2.00 µL
RT Master Mix	14.00 µL
Total Volume (µL)	36.0 µL

[1]For best results use the entire 20 µL tagged library.
[2]RT Primer included with primer sets available from TriLink.

5. Add 14 µL of master mix to each reaction tube to meet final volume shown in Table 7. Mix thoroughly by pipetting up and down, and pulse spin to collect liquid.
6. Place in thermocycler with a heated lid at 50°C for 1hr.

Optional Stop Point: If not proceeding immediately to Step 4: PCR Amplification of RT Product, cap the tube and heat deactivate at 65°C for 20 minutes, then store at- 20°C. To resume, thaw frozen samples on ice.

STEP 4: PCR Amplification of RT Product

Primers are included with the Index Primer Sets for Illumina or Barcode Convert Primer Sets for Ion Torrent (as selected by user).

1. Thaw appropriate primers and then place on ice. Keep High Fidelity PCR Master Mix on ice and do not vortex.

Note: White precipitate in master mix is common. Gently pipette up and down to resuspend mix.

2. Prepare master mix of components as shown in Table 8.

Table 8: PCR Master Mix Set Up

Component	Volume per Reaction	Volume for 3 Reactions with 10% excess
High Fidelity PCR Master Mix	40.0 µL	132 µL
Forward Primer or Barcode Convert Reverse Primer[1]	2.0 µL	6.6 µL
Total Volume	42.0 µL	138.6 µL

[1]Primers included with index primer sets. For Illumina use Forward primer; for Ion Torrent use Barcode Convert Reverse Primer.

Table 9: PCR Reaction Volume

Component	Volume per Reaction
RT Reaction Product	36.0 µL
Master Mix (from table 8)	42.0 µL
Index Primer or Barcode Convert Primer[2]	2.0 µL
Total Volume	80.0 µL
Total Volume (µL)	36.0 µL

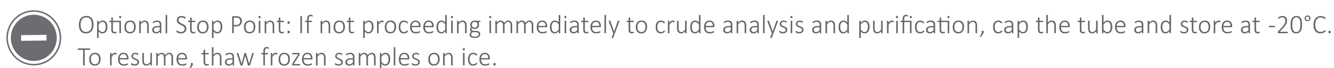
[2]Primers included with index primer sets. For Illumina use selected Index Primer; for Ion Torrent use selected Barcode Convert Primer.

3. As shown in Table 9, add 2.0 µL of the Index Primer or Barcode Convert Primer and 42 µL of master mix from Table 8 to RT Reaction Product from STEP 3 (use all product for best results). Mix well by pipetting up and down then briefly pulse spin to collect liquid.

4. Place into a thermal cycler with a heated lid. Perform the following cycling conditions using the recommended number of PCR cycles (*n*) in Table 10:
   98°C for 30 sec,
   [10 sec at 98°C; 30 sec at 60°C; 15 sec at 72°C],
   72°C for 10 min, hold at 4°C

Table 10: RNA Input PCR Cycles

RNA Input	Recommended PCR Cycles (n)
1,000 ng	12
100 ng	12-15
10 ng	18
1 ng	21



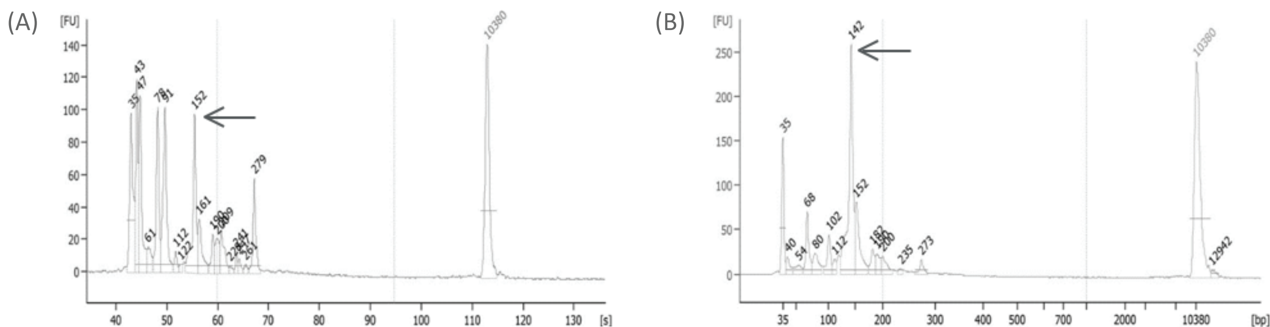
Optional Stop Point: If not proceeding immediately to crude analysis and purification, cap the tube and store at -20°C. To resume, thaw frozen samples on ice.

STEP 5: Crude Sample Analysis (Recommended)

Crude analysis of libraries is recommended to both ensure adequate libraries were prepared and help determine which purification method is best. We highly recommend using the High Sensitivity DNA Chip for Agilent 2100 Bioanalyzer for crude analysis. Depending on how much library was generated a dilution of 1:10 (higher inputs) or 1:3 (lower inputs) may be necessary before bioanalyzer, otherwise follow manufacturer's instructions.

NOTE: Crude libraries may shift ~10bp on High Sensitivity chip analysis due to remaining reaction components. For example, after purification, a peak at 152bp in a crude analysis should shift back to a more accurate size of 142bp. A peak at 142bp represents a tagged small RNA of ~21nt. See Figure 1 below.

Figure 1: Typical Bioanalyzer® results of a diluted crude sample (A) and after AMPure® XP magnetic bead-based purification (B).



STEP 6: Purification

Materials not included.
There are multiple cleanup methods available following library preparation. Depending on your library profile as determined by crude analysis and experimental goals, choose which option is best for you. Use the following table as a guide for determining your purification method based on your needs.

Table 7: Recommended Purification Options

Library Profile	Purification Method
Undesired peaks before and after 140bp library (ex: miRNA-Seq project only but other species were tagged)	2-step AMPure Bead Clean Up (OPTION A, Page 8) Gel Extraction[1] (OPTION C, Page 9) Pippin Prep (OPTION D, Page 9)
All peaks above 100bp desired (ex: sRNA-Seq project or very clean input sample type)	1-step AMPure (OPTION B, Page 9) Purelink Column (OPTION E, Page 9) Gel Extraction (OPTION C, Page 9) Pippin Prep (OPTION D, Page 9)
Small amounts of dimer peaks formed (ex: low inputs, low quality starting RNA)	Gel Extraction1 (OPTION C, Page 9) Pippin Prep (OPTION D, Page 9)

OPTION A: 2-Step AMPure® Purification (~244 µL per library sample)

NOTE: Be sure to bring AMPure XP beads to room temperature and vortex well for a homogeneous mixture before use.

This magnetic bead purification protocol may be used to replace gel purification. This protocol uses two different binding conditions to remove both higher and lower molecular weight species to size select for small RNA libraries between 100-200 base pairs.

1. Transfer PCR Product from STEP 4 to 1.5mL microcentrifuge tube to accommodate additional volumes.
2. Add 1X AMPure® XP beads (80 µL) to 80 µL PCR product. Vortex to mix thoroughly and briefly pulse spin to collect liquid.
3. Incubate at room temperature for 10 minutes.
4. Place tubes on magnetic rack for 4 minutes. Beads will collect on side of tube near magnet and supernatant will be clear.
5. Keep tubes on magnetic rack and transfer entire supernatant to clean new tube without disrupting magnetic beads on side of tube. (Supernatant contains library, discard beads.)
6. Add 1.8X AMPure® XP beads of original volume (144 µL) to supernatant. Vortex to mix thoroughly and briefly pulse spin to collect liquid.
7. Incubate for 10 minutes at room temperature off magnetic rack.
8. Place tubes on magnetic rack for 4 minutes.
9. Keep tubes on magnetic rack and aspirate off supernatant without disrupting bead pellet. Discard supernatant. (100-200 bp library is now bound to beads.)
10. Wash beads on magnetic rack with 500 µL 70% ethanol. Collect wash after 30 seconds and discard appropriately. Repeat for a total of two washes.
11. After second wash, remove all traces of ethanol and air-dry beads on rack for 5 minutes. Do not over-dry beads as elution recovery may decrease.
12. To elute DNA, resuspend AMPure® XP beads in 17 µL nuclease-free water (or Tris elution buffer) and incubate for 2 minutes at room temperature off rack.
13. Place tubes on magnetic rack for 2 minutes.
14. Being careful not to collect any beads, transfer 15 µL of supernatant (clean library) to a new clean tube.

OPTION B: 1-Step AMPure® XP Bead Purification (~144 µL per library sample)

NOTE: Be sure to bring AMPure XP beads to room temperature and vortex well for a homogeneous solution before use.

This magnetic bead purification protocol is comparable to a PCR cleanup which eliminates products smaller than 100 bp. Transfer PCR samples to 1.5mL microcentrifuge tube to accommodate additional volumes.

1. Add 1.8X AMPure® XP beads of original volume (144 µL) to PCR Sample from STEP 4. Vortex to mix thoroughly and briefly pulse spin to collect liquid.
2. Incubate for 10 minutes at room temperature off magnetic rack.
3. Place tubes on magnetic rack for 4 minutes.
4. Keep tubes on magnetic rack and aspirate off supernatant without disrupting bead pellet. Discard supernatant. (Library is now bound to beads.)
5. Wash beads on magnetic rack with 500 µL 70% ethanol. Collect wash after 30 seconds and discard appropriately. Repeat for a total of two washes.
6. After second wash, remove all traces of ethanol and air-dry beads on rack for 5 minutes. Do not over-dry as elution recovery may decrease.
7. To elute DNA, resuspend AMPure® XP beads in 17 µL nuclease-free water (or Tris elution buffer) and incubate for 2 minutes at room temperature off rack.
8. Place tubes on magnetic rack for 2 minutes.
9. Being careful not to collect any beads, transfer 15 µL of supernatant (clean library) to a new clean tube.

OPTION C: Gel Extraction Using 4% Agarose or 6% PAGE.

10. Prepare samples for gel electrophoresis. If multiplexing, mix samples equally into a pool before loading on gel. Depending on your gel, the pooled mixture may need to be split across lanes to accommodate for volume.
11. Load gel with samples leaving blank lanes on either side of library to avoid cross contamination from other wells. Run appropriate size marker on gel to determine extraction point (ex: 50bp ladder Invitrogen).
12. Run gel until sample dye is 75% through gel. Visualize with appropriate eye protection using long wavelength UV and excise library band using scalpel, do not overexpose DNA to UV light.
13. Purify gel slice using Zymoclean Gel DNA Recovery kit (or similar alternative) following manufacturer's instructions.

OPTION D: Pippin Prep Library Purification

Follow Manufacturer's instructions.

OPTION E: PureLink Column Based Purification

Follow Manufacturer's instructions.

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