iGeneTech
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For Illumina and MGI platform.

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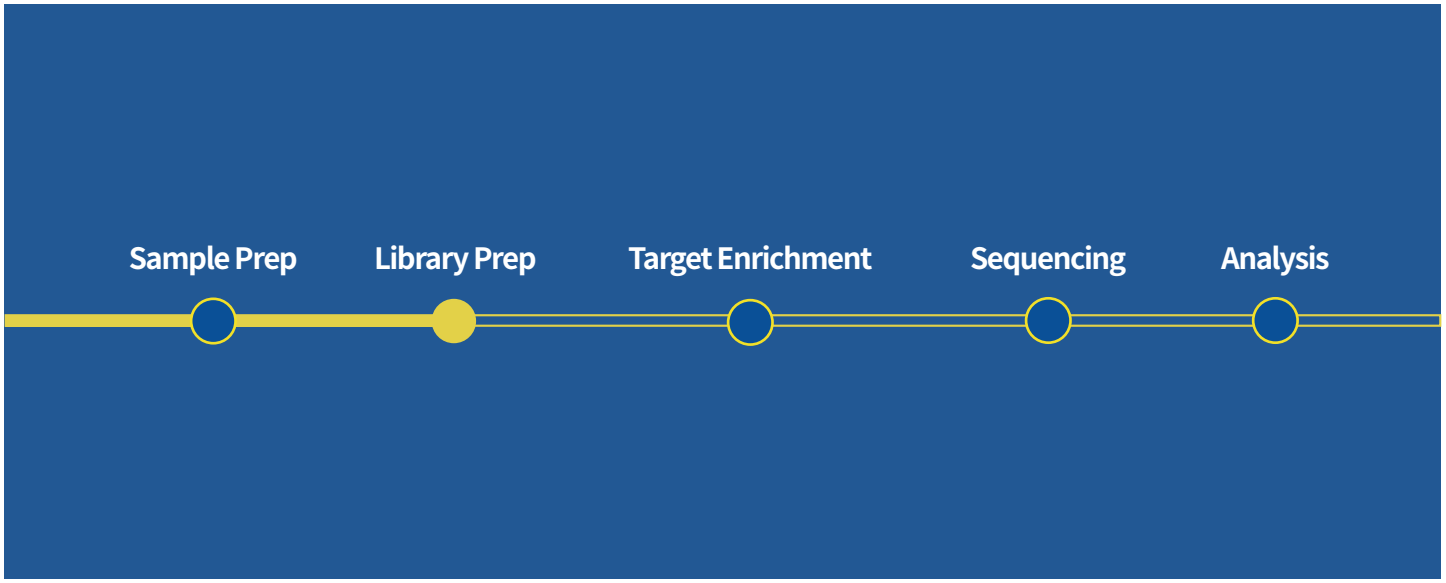
IGT® Strand-Specific RNA Library Prep Workflow v2.0

Protocol

For use with:

- IGT® Fast Stranded RNA Library Prep Kit v2.0

English Version
Version: A.0(E), August 2023
Document Number: PROT230801



Version Notes

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Protocol Notes

This protocol provides an instructions for library construction using IGT® Fast Stranded RNA Library Prep Kit v2.0. Before you begin, please read this protocol carefully, and strictly follow the protocol for experiment.

Product Notes

IGT® Fast Stranded RNA Library Prep Kit v2.0 is used for strand-specific RNA library preparation and generates sequencing libraries of Illumina and MGI platform.

Revision History

Version	Date	Description
A.0(E)	August 2023	Initial release

Materials from iGeneTech

IGT® Fast Stranded RNA Library Prep Kit v2.0 and IGT® Adapter & Primer are needed for library constrcution.

IGT® Fast Stranded RNA Library Prep Kit v2.0

Screw Cap	Components	Amount		Storage
		16 rxn	96 rxn	
Red	Fast Frag Buffer	72 µL	432 µL	-20°C ± 5°C
Brown	Fast First Strand Buffer	108 µL	640 µL	
Green	Fast First Strand Enzyme	36 µL	216 µL	
Orange	Fast Second Strand Buffer with dUTP	540 µL	2*1584 µL	
Orange	Fast Second Strand Enzyme	88 µL	540 µL	
Blue	Fast Ligation Buffer	540 µL	2*1584 µL	
Blue	Fast Ligase Mix	88 µL	540 µL	
White	PCR Master Mix with UDG	450 µL	2*1350 µL	

IGT® Adapter & Primer

Please select one of the following IGT® Adapter & Primer according to requirement.

IGT® Adapter & Single-Indexed Primer (for MGI)

Screw Cap	Components	Amount	Storage
		96*1 rxn	
Blue	Adapter (15 µM, for MGI SI)	540 µL	-20°C ± 5°C
White	TPE 1.0 Primer (20µM, for MGI)	264 µL	
/	TPE 2.0 Indexed Primer N (20µM, for MGI)*	4 µL each	

*N is the index number.

IGT® Adapter & UDI Primer (for Illumina/MGI)

Screw Cap	Components	Amount		Storage
		96*1 rxn	96*10 rxn	
Blue	Adapter (15 µM)	540 µL	4*1350 µL	-20°C ± 5°C
/	UDI Primer N (10 µM each)*	8 µL each	96*75 µL	

*N is the index number.

IGT® UMI Adapter & UDI Primer (for Illumina/MGI)

Screw Cap	Components	Amount		Storage
		96*1 rxn	96*10 rxn	
Blue	Insert UMI Adapter (15 µM)	540 µL	4*1350 µL	-20°C ± 5°C
/	UDI Primer N (10 µM each)*	8 µL each	96*75 µL	

*N is the index number.

Materials Supplied by User

The materials below are recommended by iGeneTech. Please select the appropriate materials to start the experiment.

Reagents

Item	Recommended reagents	Supplier (Cat #)
Ethanol Absolute	Varies	Varies
Nuclease-Free Water	Nuclease-Free Water	Ambion (AM9930)
Magnetic Beads*	Agencourt AMPure XP Kit	Beckman Coulter (A63880)
	IGT® Pure Beads	iGeneTech (C80661)
Fragment Analyzer	Agilent DNA 1000 Kit	Agilent (5067-1504)
Nucleic Acid Quantification Assays	Qubit dsDNA HS Assay Kit	Thermo Fisher (C47257)

*Choose one of the recommended reagents.

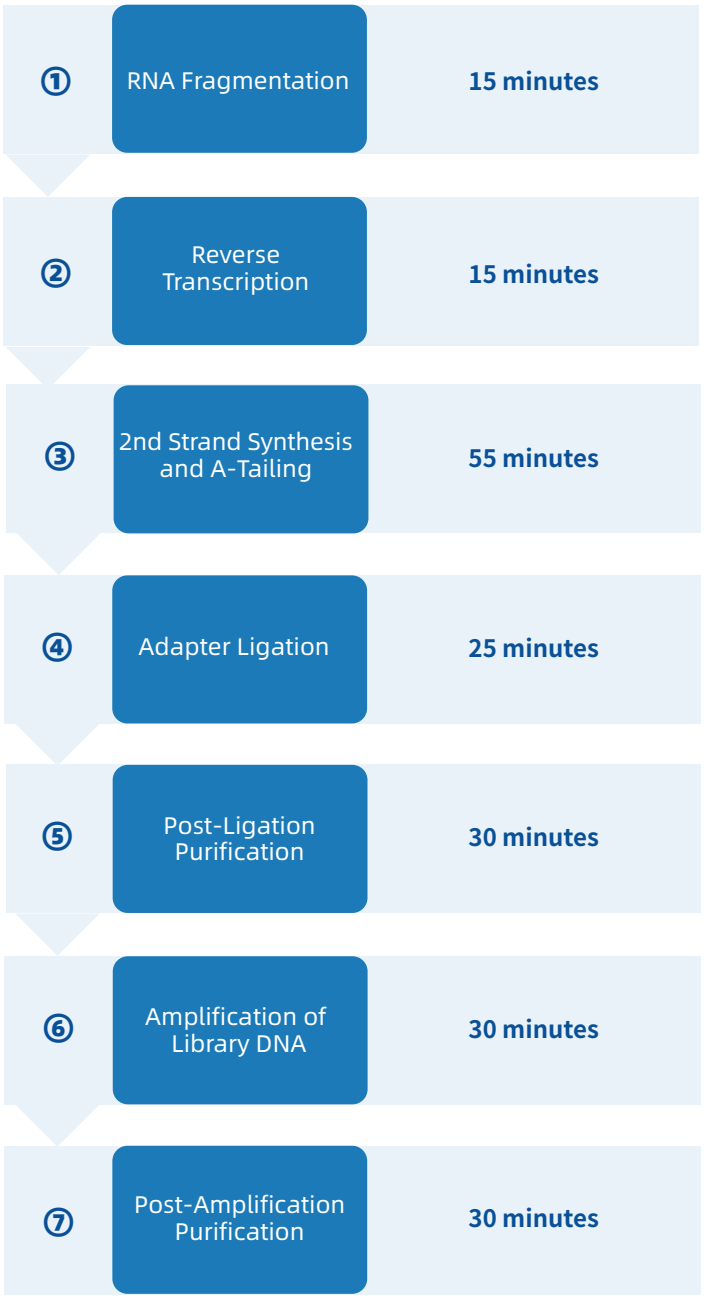
Equipment

Item	Recommended equipments	Supplier (Cat #)
96-well Magnetic Stand, 0.2 mL block	DynaMag-96 Side	Thermo Fisher (12331D)
Fragment Analyzer	Agilent 2100 Bioanalyzer system	Agilent (G2939AA)
Nucleic Acid Quantification Instrument	Qubit® 4.0 Fluorometer	Thermo Fisher (Q33238)
Vortex Mixer	Varies	Varies
Mini Centrifuge	Varies	Varies
Ice Block	Varies	Varies
Thermal Cycler	Varies	Varies

Consumables

Item	Recommended consumables	Supplier (Cat #)
Qubit tubes, 0.5 mL	Qubit® assay tubes	Thermo Fisher (Q32856)
PCR tubes, 0.2 mL	Varies	Varies
8-tubes strip, 0.2 mL	Varies	Varies
Pipette tips, 10 µL	Varies	Varies
Pipette tips, 200 µL	Varies	Varies

Workflow


①
RNA Fragmentation
15 minutes
②
Reverse Transcription
15 minutes
③
2nd Strand Synthesis and A-Tailing
55 minutes
④
Adapter Ligation
25 minutes
⑤
Post-Ligation Purification
30 minutes
⑥
Amplification of Library DNA
30 minutes
⑦
Post-Amplification Purification
30 minutes

Before You Begin

To start, please confirm the following requirements are met.

- ☐ Please use RNase-Free equipment during the library construction process.
- ☐ Before the experiment, please confirm whether the self-prepared reagents (such as absolute ethanol) meet the experimental conditions and order them as needed.
- ☐ Some experimental steps during the experiment cannot be suspended, please follow the specific operation instructions to carry out the experiment.
- ☐ Buffers, primers and other reagents preserved at -20 ° C must be thawed on ice and fully mixed before use. All these cannot be dissolved by high-temperature heating and other methods.
- ☐ If the reagent amount used in each experiment is small, aliquot is recommended to avoid quality changes caused by repeated freeze-thaw.
- ☐ Minimize the number of freeze-thaw cycles of samples to avoid RNA degradation which leads to small RNA fragments and poor sequencing quality.
- ☐ The sample quality has a great influence on experimental results, and it is recommended to use qualified samples for experiments.
- ☐ Please use enzyme/enzyme mix immediately after pulse-spin down (⩽ 600 g).
- ☐ Please take out the magnetic beads from 4° C freezer and incubate at room temperature for 30 minutes before use.
- ☐ A260/A280 = 1.8 to 2.1 is generally accepted as "pure" for RNA samples. If the ratio is lower/higher, it may indicate the presence of protein or genomic DNA.
- ☐ Wear gloves and mask when handling reagents and preparing RNA libraries. Keep RNA samples on ice and the lab environment clean to avoid RNA degradation.

The experiment can be started if the criterias above are met.

STEP 1 RNA Fragmentation

Experiment Notes

Regents required:

· Fast Frag Buffer

Equipment required:

· Thermal Cycler

1.1 Thaw RNA samples and Fast Frag Buffer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

1.2 Prepare reaction mixture on ice as indicated below.

Component	Volume
RNA	13 µL (10 ng~1 µg in total)
Fast Frag Buffer	4 µL
Total Volume	**17 µL**

1.3 Mix well by gently pipetting or votexing and centrifuge briefly.

1.4 Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
	Heat lid temperature 105°C
94°C	7 min
4°C	Hold

1.5 When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 2 immediately.

This is not a stop point, please proceed to STEP 2 immediately.

STEP 2 Reverse Transcription

Experiment Notes

Regents required:

- Fast First Strand Buffer
- Fast First Strand Enzyme

Equipment required:

- Thermal Cycler

2.1 Thaw Fast First Strand Buffer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

2.2 Place First Strand Enzyme on ice. Mix well by rotating and then centrifuge briefly. Place back on ice.

2.3 Prepare reaction mixture on ice as indicated below.

Component	Volume
Sample from STEP 1.5	17 µL
Fast First Strand Buffer	6 µL
Fast First Strand Enzyme	2 µL
Total Volume	**25 µL**

2.4 Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

2.5 Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
Heat lid temperature 105°C	
25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

2.6 When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 3 immediately.

This is not a stop point, please proceed to the STEP 3 immediately.

STEP 3 2nd Strand Synthesis & A-Tailing

Experiment Notes

Regents required:

- Fast Second Strand Buffer with dUTP
- Fast Second Strand Enzyme

Equipment required:

- Thermal Cycler

3.1 Thaw Fast Second Strand Buffer with dUTP on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

3.2 Place Fast Second Strand Enzyme on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

3.3 Prepare reaction mixture on ice as indicated below.

Component	Volume
Sample from STEP 2.6	25 µL
Fast Second Strand Buffer with dUTP	30 µL
Fast Second Strand Enzyme	5 µL
Total Volume	**60 µL**

3.4 Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

3.5 Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
Heat lid temperature 105°C	
16℃	30 min
72℃	15 min
4℃	Hold

3.6 When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 4 immediately.

This is not a stop point, please proceed to the STEP 4 immediately.

STEP 4 Adapter Ligation

Experiment Notes

Regents required:

- Adapter
- Fast Ligation Buffer
- Fast Ligase Mix

Equipment required:

- Thermal Cycler

4.1 Thaw Adapter on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

4.2 Dilute the Adapter (15 μM) according to the table below.

RNA input	Adapter concentration	Dilution times
100 ng ~ 500 ng	7.5 μM	2
50 ng	3.75 μM	4
25 ng	1.5 μM	10
10 ng	0.75 μM	20

4.3 Thaw Fast Ligation Buffer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

4.4 Place Fast Ligase Mix on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

4.5 Prepare reaction mixture on ice as indicated below.

Component	Volume
Sample from STEP 3.6	60 μL
Fast Ligation Buffer	30 μL
Fast Ligase Mix	5 μL
Adapter (Diluted)	5 μL
Total Volume	**100 μL**

If handling many samples at the same time, prepare the reaction mixture without Adapter. It is suggested to mix the Adapter and the products from STEP 3.6 first, and then add the reaction mixture to reduce adapter self-ligation.

4.6 Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

4.7 Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
	Shut down heat lid
20℃	15 min
4℃	Hold

4.8 When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 5 immediately.

This is not a stop point, please proceed to the STEP 5 immediately.

STEP 5 Post-Ligation Purification

Experiment Notes

Regents required:

- Magnetic Beads
- 80% Ethanol (freshly prepared)
- Nuclease-Free Water

Equipment required:

- Magnetic Stand

IGT® Pure Beads and Agencourt AMPure XP are the recommended magnetic beads in this protocol. Please chose the suitable volume according to the library type. Please assess the volume of beads used for purification via pre-experiments if not using the recommended one.

5.1 Prepare the 80% ethanol for purification. Place the 80% ethanol at room temperature.

5.2 Vortex the magnetic beads for 30 sec to mix well, and equilibrate the magnetic beads to room temperature for 30 min.

5.3 Add 45 µL (0.45×) of magnetic beads to each ligated sample from STEP 4.8. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

5.4 Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 3 min).

5.5 Keep the PCR tube containing beads on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec.

5.6 Keep the PCR tube containing beads on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec. Remove and discard the clear supernatant.

5.7 Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant. Remove all traces of supernatant using a 10 µL pipette.

5.8 Keep the PCR tube containing beads on the magnetic stand at room temperature for 3 ~ 5 min to dry the bead pellet. Do not overdry the bead pellet.

5.9 Add 22 µL of Nuclease-Free Water. Remove the PCR tube containing beads from the magnetic stand. Vortex for 10 sec or pipet to mix well. Incubate at room temperature for 2 min.

5.10 Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 2 min).

5.11 Transfer 20 µL of clear supernatant containing each library to a clean PCR tube. Please proceed to STEP 6.

This is not a stop point, please proceed to the STEP 6 immediately.

STEP 6 Amplification of Library DNA

Experiment Notes

Regents required:

- PCR Master Mix with UDG
- UDI Primer

Equipment required:

- Thermal Cycler

6.1 Thaw PCR Master Mix with UDG on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

6.2 Thaw UDI Primer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

6.3 Prepare PCR reaction mixture on ice as indicated below.

Component	Volume per reaction
Sample from STEP 5.11	20 µL
PCR Master Mix with UDG	25 µL
UDI Primer N (10 µM each)	5 µL
Total Volume	**50 µL**

Make sure the index number N of indexed primer are marked down.

6.4 Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

6.5 Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temprture	Time	PCR cycles
Heat lid temperature 105°C		
98℃	1 min	1
98℃	10 s	N cycles
60℃	30 s	
72℃	30 s	
72℃	5 min	1
4℃	Hold	

DNA input	PCR cycles N (1 µg library output)
500 ng	7
250 ng	7
100 ng	9
50 ng	10
25 ng	11
10 ng	12

PCR cycle number is adjusted according to RNA input.

6.6 When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 7 immediately.

This is not a stop point, please proceed to the STEP 7 immediately.

STEP 7 Post-Amplification Purification

Regents required:

- Magnetic Beads
- 80% Ethanol (freshly prepared)
- Nuclease-Free Water

Equipment required:

- Magnetic Stand

IGT® Pure Beads and Agencourt AMPure XP are the recommended magnetic beads in this protocol. Please chose the suitable volume according to the library type. Please assess the volume of beads used for purification via pre-experiments if not using the recommended one.

7.1 Prepare the 80% ethanol for purification. Place the 80% ethanol at room temperature.

7.2 Vortex the magnetic beads for 30 sec to mix well. Equilibrate the beads to room temperature for 30 min.

7.3 Add 45 µL (0.9×) of magnetic beads to each amplified sample from STEP 6.6. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

7.4 Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 3 min).

7.5 Keep the PCR tube containing beads on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec.

7.6 Keep the PCR tube containing beads on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec. Remove and discard the clear supernatant.

7.7 Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant. Remove all traces of supernatant using a 10 µL pipette.

7.8 Keep the PCR tube containing beads on the magnetic stand at room temperature for 3 ~ 5 min to dry the bead pellet. Do not overdry the bead pellet.

7.9 Add 30 µL of Nuclease-Free Water. Remove the PCR tube containing beads from the magnetic stand. Vortex for 10 sec or pipet to mix well. Incubate at room temperature for 2 min.

7.10 Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant.

7.11 Transfer 28 µL of clear supernatant containing each library to a clean PCR tube. Libraries can be stored at -20℃ for up to one month.

7.12 Quantify each library using Qubit dsDNA High Sensitivity Assay Kit. Analyze the fragment size of each library using Agilent 2100 Bioanalyzer.

The experiment ends here.

Experiment Notes

Note

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