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

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Protocol status: Working
We use this protocol and it's working

ABSTRACT

In this protocol, we provide sequences of oligos used for high-throughput sequencing (HTS) applications and describe methods for preparing the oligos for HTS applications. This protocol (including oligo sequences and oligo preparation) is based on previously described methods with minor modifications (Foley et al., 2019; Glenn et al., 2019; and Veeranagouda et al., 2019).

REFERENCES

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Veeranagouda Y, Remaury A, Guillemot JC, Didier M (2019). RNA Fragmentation and Sequencing (RF-Seq): Cost-Effective, Time-Efficient, and High-Throughput 3' mRNA Sequencing Library Construction in a Single Tube. *Current Protocols in Molecular Biology*, *129*(1). https://doi.org/10.1002/cpmb.109

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GUIDELINES

Standard laboratory guidelines and practices should be followed when performing this protocol.

MATERIALS

Reagents:

- Nuclease-free water
- MilliQ water

Solutions:

- 1 M Tris-HCl (pH 8.0)
- 0.5 M EDTA (pH 8.0)
- 5 M NaCl

Consumables:

- 1.5 ml microcentrifuge tubes (nuclease-free)
- 1.5 ml screw-cap microcentrifuge tubes (nuclease-free)
- 0.2 ml PCR tubes or strips (low-bind, nuclease-free)

Oligos:

- Y-yoke adapter oligos

	A	B
	Name	Sequence
	iTrusR2-stubRCp	/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCA*C
	iTrusR1-stub	ACACTCTTTCCCTACACGACGCTCTTCCGATC*T

Note

Y-yoke adapter oligos are based on Glenn et al., 2019. "/5Phos/" indicates 5´ phosphorylation. "" indicates a phosphorothioate bond. Standard desalting is sufficient.*

- Library barcoding/enrichment oligos

	A	B	C
	Name	Sequence	Barcode
	i5-D501	AATGATACGGCGACCACCGAGATCTACACAGGCTATAACACTCTTTCCCTACACGACGCTCTTCCGATC*T	AGGCTATA
	i5-D502	AATGATACGGCGACCACCGAGATCTACACGCCTCTATACACTCTTTCCCTACACGACGCTCTTCCGATC*T	GCCTCTAT
	i5-D503	AATGATACGGCGACCACCGAGATCTACACAGGATAGGACACTCTTTCCCTACACGACGCTCTTCCGATC*T	AGGATAGG
	i5-D504	AATGATACGGCGACCACCGAGATCTACACTCAGAGCCACACTCTTTCCCTACACGACGCTCTTCCGATC*T	TCAGAGCC

	A	B	C
	i5-D505	AATGATACGGCGACCACCGAGATCTACACCTTCGCCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T	CTTCGCCT
	i5-D506	AATGATACGGCGACCACCGAGATCTACACTAAGATTAACACTCTTTCCCTACACGACGCTCTTCCGATC*T	TAAGATTA
	i5-D507	AATGATACGGCGACCACCGAGATCTACACACGTCCTGACACTCTTTCCCTACACGACGCTCTTCCGATC*T	ACGTCCTG
	i5-D508	AATGATACGGCGACCACCGAGATCTACACGTCAGTACACACTCTTTCCCTACACGACGCTCTTCCGATC*T	GTCAGTAC
	i7-D701	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CGAGTAAT
	i7-D702	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TCTCCGGA
	i7-D703	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	AATGAGCG
	i7-D704	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GGAATCTC
	i7-D705	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TTCTGAAT
	i7-D706	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACGAATTC
	i7-D707	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGGTTCAGACGTGTGCTCTTCCGATC*T	AGCTTCAG
	i7-D708	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GCGCATTA
	i7-D709	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CATAGCCG
	i7-D710	CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGGTTCAGACGTGTGCTCTTCCGATC*T	TTCGCGGA
	i7-D711	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GCGCGAGA
	i7-D712	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CTATCGCT

Note

Library barcoding/enrichment oligos are based on Veeranagouda et al., 2019. i7-D701-D712 primers differ in eight bases as indicated in bold letters. "" indicates a phosphorothioate bond. Standard desalting is sufficient.*

- 3´ mRNA capture oligos

	A	B
	Name	Sequence
	IRA_UMI_24dTVN	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNVVVVTTTTTTTTTTTTTTTTTTTTTTVN
	IFA_isoTSO	/5Biosg/CCACACTCTTTCCCTACACGACGCTCTTCCGATCTrGrGrG

Note

3´ mRNA capture oligos are based on Foley et al., 2019 and Veeranagouda et al., 2019. "/5Biosg/" indicates 5´ Biotinylation. A base proceeded by a lower case "r" indicates an RNA base. The IRA_UMI_24dTVN oligo is PAGE purified. Standard desalting is sufficient for the IFA_isoTSO oligo.

SAFETY WARNINGS

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

Preparation of Y-yoke adapters

1 Prepare the following buffer in a 50 ml conical tube:

TLEN (Tris-HCl, Low EDTA, NaCl) buffer

	A	B	C
	Reagent	Volume	Final conc.
	1 M Tris-HCl (pH 8.0)	500 µl	10 mM
	0.5 M EDTA (pH 8.0)	10 µl	0.1 mM
	5 M NaCl	1 ml	100 mM
	MilliQ water	48.49 ml	–
	Total	50 ml	–

Note

TLEN buffer can be prepared in advance and stored at room temperature. The preparation of Y-yoke adapters and TLEN buffer is based on Glenn et al., 2019.

2 Briefly centrifuge the lyophilized adapter oligos at room temperature.

3 Prepare a 100 µM stock of each adapter oligo. Add TLEN buffer at a volume 10 times the nmol of the oligo. For example, if the quantity of a given adapter oligo is nmol, add 1265 µl of TLEN buffer.

4 Vortex the adapter oligo suspensions on setting 4 for 2 s, incubate at room temperature for 5 min, and briefly centrifuge at room temperature.

5 Repeat Step 4 once to ensure adapter oligos are fully resuspended.

6 Combine equal volumes of each adapter oligo in a 1.5 ml microcentrifuge tube, vortex on setting 4 for 5 s, and briefly centrifuge at room temperature.

Note

Combining equal volumes of each adapter oligo yields an equal molar solution at 50 µM for each adapter oligo (e.g., combining 50 µl of each adapter oligo yields a 100 µl solution containing each adapter oligo at a concentration of 50 µM).

7 Split the adapter oligo mixture into 50 µl aliquots in 0.2 ml PCR tubes or strips.

8 Anneal the adapter oligos in a thermocycler with heated lid using the following conditions: 95°C for 2 min, 73 cycles of cooling (minus 1°C per min) to reach 21°C, and 20°C for 10 min.

9 Pool aliquots of annealed adapter oligos (50 µM) in a 1.5 ml screw-cap microcentrifuge tube, vortex on setting 4 for 5 s, and briefly centrifuge at room temperature. This pool is a 50 µM stock of annealed adapters.

10 Prepare 15 µM, 1.5 µM, and 0.15 µM stocks of the annealed adapters in TLEN buffer by serial dilution of the 50 µM stock.

11 Split the annealed adapter stocks into 50 µl aliquots in 1.5 ml screw-cap microcentrifuge tubes and store at -20°C.

Note

Annealed adapters lose effectiveness after multiple freeze-thaw cycles. Small aliquots of annealed adapters help minimize the number of freeze-thaw cycles.

12 Shortly before use, thaw annealed adapter stocks on ice, vortex on setting 4 for 1 s, and briefly centrifuge at room temperature. Avoid freeze-thaw cycles when possible.

Preparation of library barcoding/enrichment oligos and mixes of oligo pairs

13 Prepare the following buffer in a 50 ml conical tube:

TLE (Tris-HCl, Low EDTA) buffer

	A	B	C
	Reagent	Volume	Final conc.
	1 M Tris-HCl (pH 8.0)	500 µl	10 mM

	A	B	C
	0.5 M EDTA (pH 8.0)	10 µl	0.1 mM
	MilliQ water	49.49 ml	–
	Total	50 ml	–

Note

TLE buffer can be prepared in advance and stored at room temperature. The preparation of TLE buffer is based on Glenn et al., 2019.

14 Briefly centrifuge the lyophilized library oligos at room temperature.

15 Prepare a 100 µM stock of each library oligo. Add TLE buffer at a volume 10 times the nmol of the oligo. For example, if the quantity of a given adapter oligo is nmol, add 590 µl of TLE buffer.

16 Vortex the library oligo suspensions on setting 4 for 2 s, incubate at room temperature for 5 min, and briefly centrifuge at room temperature.

17 Repeat Step 16 once to ensure library oligos are fully resuspended.

18 Prepare 10 µM primer pair mixtures. Combine 10 µl of a given i5 library oligo, 10 µl of a given i7 library oligo, and 180 µl of TLE buffer in a 1.5 ml screw-cap microcentrifuge tube.

Note

In the 10 µM primer pair mixture, the total oligo concentration is 10 µM and the concentration of individual oligo is 5 µM. See Step 30 for information on primer pairs.

19 Vortex 10 µM primer pair mixtures on setting 4 for 5 s and briefly centrifuge at room temperature.

20 Prepare 5 µM primer pair mixtures. Combine 50 µl of a given 10 µM primer pair mixture and 50 µl of TLE buffer in a 1.5 ml screw-cap microcentrifuge tube.

Note

In the 5 µM primer pair mixture, the total oligo concentration is 5 µM and the concentration of individual oligo is 2.5 µM. See Step 30 for information on primer pairs.

21 Vortex 5 µM primer pair mixtures on setting 4 for 5 s and briefly centrifuge at room temperature.

22 Store primer pair mixtures at -20°C.

23 Shortly before use, thaw primer pair mixtures on ice, vortex on setting 4 for 1 s, and briefly centrifuge at room temperature. Avoid freeze-thaw cycles when possible.

Preparation of 3´ mRNA capture oligos

24 Briefly centrifuge the lyophilized capture oligos at room temperature.

25 Prepare a 100 µM stock of each capture oligo. Add nuclease-free water at a volume 10 times the nmol of the oligo. For example, if the quantity of a given capture oligo is 1 nmol, add 101 µl of nuclease-free water.

26 Vortex the capture oligo suspensions on setting 4 for 2 s, incubate on ice for 10 min, and briefly centrifuge at room temperature.

27 Repeat Step 26 once to ensure capture oligos are fully resuspended.

28 Prepare 20 µM stocks of the capture oligos in nuclease-free water in 1.5 ml screw-cap microcentrifuge tubes and store at -20°C.

Note

Capture oligos lose effectiveness after multiple freeze-thaw cycles. Small aliquots of capture oligos help minimize the number of freeze-thaw cycles and reduce the risk of RNase contamination.

29 Shortly before use, thaw capture oligo stocks on ice, vortex on setting 4 for 1 s, and briefly centrifuge at room temperature. Avoid freeze-thaw cycles when possible.

Strategy for pairing and selecting of library barcoding/enrichment oligos

30 Use the following schematic to select pairs of library barcoding/enrichment oligos to use for a given project.

Note

Pairing and selecting library barcoding/enrichment oligos is based on Veeranagouda et al., 2019 and Glenn et al., 2019.

Pairing and selecting oligos for library barcoding/enrichment. Each sample should contain a unique pair of oligos. Demultiplexing requires preservation of “sequence diversity” among barcodes. Choose combinations of oligo pairs as indicated above based on the number of samples. As an example, use oligo pairs in the blue, orange, or green rectangles when preparing libraries from 4, 12, or 16 samples, respectively. When preparing libraries from a larger number of samples, select additional oligo pairs using a similar strategy. Schematic is modified from Veeranagouda et al., 2019.