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

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We use this protocol and it's working

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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>

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	AATGATACGGCGACCACCGAGATCTACACAGGCTATAACACTCTTTCCCTA CACGACGCTCTTCCGATC*T	AGGCTATA
	i5-D502	AATGATACGGCGACCACCGAGATCTACACGCCTCTATACACTCTTTCCCTA CACGACGCTCTTCCGATC*T	GCCTCTAT
	i5-D503	AATGATACGGCGACCACCGAGATCTACACAGGATAGGACACTCTTTCCCTA CACGACGCTCTTCCGATC*T	AGGATAG G



	A	B	C
	i5-D504	AATGATACGGCGACCACCGAGATCTACACTCAGAGCCACACTCTTTCCCTA CACGACGCTCTTCCGATC*T	TCAGAGC C
	i5-D505	AATGATACGGCGACCACCGAGATCTACACCTTCGCCTACACTCTTTCCCTA CACGACGCTCTTCCGATC*T	CTTCGCC T
	i5-D506	AATGATACGGCGACCACCGAGATCTACACTAAGATTAACACTCTTTCCCTA CACGACGCTCTTCCGATC*T	TAAGATTA
	i5-D507	AATGATACGGCGACCACCGAGATCTACACACGTCCTGACACTCTTTCCCTA CACGACGCTCTTCCGATC*T	ACGTCCT G
	i5-D508	AATGATACGGCGACCACCGAGATCTACACGTCAGTACACACTCTTTCCCTA CACGACGCTCTTCCGATC*T	GTCAGTAC
	i7-D701	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGT GTGCTCTTCCGATC*T	CGAGTAAT
	i7-D702	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACG TGTGCTCTTCCGATC*T	TCTCCGG A
	i7-D703	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGT GTGCTCTTCCGATC*T	AATGAGC G
	i7-D704	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGT GTGCTCTTCCGATC*T	GGAATCT C
	i7-D705	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGT GTGCTCTTCCGATC*T	TTCTGAAT
	i7-D706	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGT GTGCTCTTCCGATC*T	ACGAATTC
	i7-D707	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGGTTCAGACGT GTGCTCTTCCGATC*T	AGCTTCA G
	i7-D708	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGT GTGCTCTTCCGATC*T	GCGCATT A
	i7-D709	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACG TGTGCTCTTCCGATC*T	CATAGCC G
	i7-D710	CAAGCAGAAGACGGCATACGAGATTTGCGGGAGTGACTGGGTTCAGACGT GTGCTCTTCCGATC*T	TTCGCGG A
	i7-D711	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACG TGTGCTCTTCCGATC*T	GCGCGAG A
	i7-D712	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGT GTGCTCTTCCGATC*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_24dTV N	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNVVVVTTTTTTT TTTTTTTTTTTTTTTTTVN
	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.

Troubleshooting

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 μ l	10 mM
	0.5 M EDTA (pH 8.0)	10 μ l	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 μ M 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 μ l 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 μ M for each adapter oligo (e.g., combining 50 μ l of each adapter oligo yields a 100 μ l solution containing each adapter oligo at a concentration of 50 μ M).

- 7 Split the adapter oligo mixture into 50 μ l 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 μ M) 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 μ M stock of annealed adapters.
- 10 Prepare 15 μ M, 1.5 μ M, and 0.15 μ M stocks of the annealed adapters in TLEN buffer by serial dilution of the 50 μ M stock.
- 11 Split the annealed adapter stocks into 50 μ l 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.



	A	B	C
	1 M Tris-HCl (pH 8.0)	500 μ l	10 mM
	0.5 M EDTA (pH 8.0)	10 μ l	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 μ M 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 μ l 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 μ M primer pair mixtures. Combine 10 μ l of a given i5 library oligo, 10 μ l of a given i7 library oligo, and 180 μ l of TLE buffer in a 1.5 ml screw-cap microcentrifuge tube.

Note

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

- 19 Vortex 10 μ M 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.



	I7-D701	I7-D702	I7-D703	I7-D704	I7-D705	I7-D706	I7-D707	I7-D708	I7-D709	I7-D710	I7-D711	I7-D712
I5-D501	Primer pair P1 D501/D701	Primer pair P2 D501/D702	Primer pair P3 D501/D703	Primer pair P4 D501/D704	Primer pair P5 D501/D705	Primer pair P6 D501/D706	Primer pair P7 D501/D707	Primer pair P8 D501/D708	Primer pair P9 D501/D709	Primer pair P10 D501/D710	Primer pair P11 D501/D711	Primer pair P12 D501/D712
I5-D502	Primer pair P13 D502/D701	Primer pair P14 D502/D702	Primer pair P15 D502/D703	Primer pair P16 D502/D704	Primer pair P17 D502/D705	Primer pair P18 D502/D706	Primer pair P19 D502/D707	Primer pair P20 D502/D708	Primer pair P21 D502/D709	Primer pair P22 D502/D710	Primer pair P23 D502/D711	Primer pair P24 D502/D712
I5-D503	Primer pair P25 D503/D701	Primer pair P26 D503/D702	Primer pair P27 D503/D703	Primer pair P28 D503/D704	Primer pair P29 D503/D705	Primer pair P30 D503/D706	Primer pair P31 D503/D707	Primer pair P32 D503/D708	Primer pair P33 D503/D709	Primer pair P34 D503/D710	Primer pair P35 D503/D711	Primer pair P36 D503/D712
I5-D504	Primer pair P37 D504/D701	Primer pair P38 D504/D702	Primer pair P39 D504/D703	Primer pair P40 D504/D704	Primer pair P41 D504/D705	Primer pair P42 D504/D706	Primer pair P43 D504/D707	Primer pair P44 D504/D708	Primer pair P45 D504/D709	Primer pair P46 D504/D710	Primer pair P47 D504/D711	Primer pair P48 D504/D712
I5-D505	Primer pair P49 D505/D701	Primer pair P50 D505/D702	Primer pair P51 D505/D703	Primer pair P52 D505/D704	Primer pair P53 D505/D705	Primer pair P54 D505/D706	Primer pair P55 D505/D707	Primer pair P56 D505/D708	Primer pair P57 D505/D709	Primer pair P58 D505/D710	Primer pair P59 D505/D711	Primer pair P60 D505/D712
I5-D506	Primer pair P61 D506/D701	Primer pair P62 D506/D702	Primer pair P63 D506/D703	Primer pair P64 D506/D704	Primer pair P65 D506/D705	Primer pair P66 D506/D706	Primer pair P67 D506/D707	Primer pair P68 D506/D708	Primer pair P69 D506/D709	Primer pair P70 D506/D710	Primer pair P71 D506/D711	Primer pair P72 D506/D712
I5-D507	Primer pair P73 D507/D701	Primer pair P74 D507/D702	Primer pair P75 D507/D703	Primer pair P76 D507/D704	Primer pair P77 D507/D705	Primer pair P78 D507/D706	Primer pair P79 D507/D707	Primer pair P80 D507/D708	Primer pair P81 D507/D709	Primer pair P82 D507/D710	Primer pair P83 D507/D711	Primer pair P84 D507/D712
I5-D508	Primer pair P85 D508/D701	Primer pair P86 D508/D702	Primer pair P87 D508/D703	Primer pair P88 D508/D704	Primer pair P89 D508/D705	Primer pair P90 D508/D706	Primer pair P91 D508/D707	Primer pair P92 D508/D708	Primer pair P93 D508/D709	Primer pair P94 D508/D710	Primer pair P95 D508/D711	Primer pair P96 D508/D712

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.