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FLAM-seq (with Kapa mRNA enrichment)_CMS_edit-2024-09-25

Forked from a private protocol

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

fork__CMS_edit-2024-09-25

Materials

Kapa mRNA HyperPre Kit Illumina (cat. KK8440, Roche; Replaces TruSeq mRNA)

- mRNA Capture Beads
- mRNA Bead Binding Buffer (BBB)
- mRNA Bead Wash Buffer(BWB)

Poly(A) Tail Length Assay Kit (cat. 764551KT, Thermo Fisher)

- 5X tail buffer mix
- 10X tail enzyme mix
- Universal Reverse (Univ. RV Primer; used in Sect 4: cDNA Amplification)

SMARTer PCR cDNA cDNA Synthesis Kit (cat. 634926, Takara)

- 5X First strand buffer
- DTT 20mM
- dNTP mix 10 mM
- RNase Inhibitor
- SMARTScribe RT
- 5' PCR Primer II A (used in Sect 4: cDNA Amplification)

Advantage 2 DNA polymerase mix (cat 639207, Takara)

- 10X Advantage 2SA PCR buffer
- dNTP Mix (10 mM each)

RNAClean XP Beads (cat. A63987, Beckman Coulter)
XP DNA beads (cat. A63881, Beckman Coulter)

Custom Oligonucleotides
isoTSO - Template switch oligo (where “i” indicates stereoisomers of dC and dG as in the associated publication):
iCiGiCAAGCAGTGGTATCAACGCAGAGTACATrGrGrG

RT primer 1:
GGTAATACGACTCACTATAGCGAGANNNNNNNNNNCCCCCCCCCTTT
PCR primer 1 (to use in combination with RT primer 1; not used; replaced by 5' PCR Primer II A):
GGTAATACGACTCACTATAGCGAG

RT primer 2 (to use in alternative to 1):
TGAGTCGGCAGAGAACTGGCGAANNNNNNNNNNCCCCCCCCCTTT,
PCR primer 2 (to use in combination with RT primer 2; not used; replaced by 5' PCR Primer II A):
TGAGTCGGCAGAGAACTGGCGAA

Poly(A)+ RNA preparation (using Kapa mRNA Beads)

10m 10s

1 Prepare mRNA Beads

1.1 52.5 µL mRNA Beads can be scaled for multiple samples
500 rpm, 00:01:00

1.2 Room temperature 00:05:00 magnet 5m
Discard supernatant

1.3 Remove from magnet
52.5 µL BBB
500 rpm, 00:01:00

1.4 Room temperature 00:05:00 magnet
Discard supernatant

1.5 Remove from magnet
52.5 µL BBB
500 rpm, 00:01:00

2 2-10 µg ds DNA in 0.2 mL PCR tube
50 µL QS; NAF

3 50 µL re-suspended mRNA Beads (from 1.5)
1000 rpm, 20°C, 00:01:00

4 65 °C 00:02:00 7m 10s
20 °C 00:05:00
Undetermined, 00:00:10 , pulse

5 Room temperature 00:05:00 magnet 5m
Discard supernatant

6 Remove from magnet
200 µL BWB
1000 rpm, 20°C, 00:01:00

7 Room temperature 00:05:00 magnet 5m
Discard supernatant

8 50 µL NAF
1000 rpm, 20°C, 00:01:00

9 70 °C 00:02:00 7m 10s
20 °C 00:05:00
Undetermined, 00:00:10 , pulse

10 50 µL BBB 5m
1000 rpm, 20°C, 00:01:00
20 °C 00:05:00

11 Room temperature 00:05:00 magnet 5m
Discard supernatant

12 Remove from magnet
200 µL BWB
1000 rpm, 20°C, 00:01:00

13 Room temperature 00:05:00 magnet 5m
Discard supernatant

14 Remove from magnet
16 µL NAF
1000 rpm, 20°C, 00:01:00

15 70 °C 00:02:00 2m

16 Room temperature 00:05:00 magnet 5m

17 16 µL ds DNA to new 0.2 mL PCR tube

GI Tailing (using USB poly(A) tail length assay, Thermo Fisher).

18 Prepare on ice (20 uL total):
14 µL RNA - poly(A)+
4 µL 5X tail buffer mix
2 µL 10X tail enzyme mix

19 37 °C 01:00:00 1h

20 1.5 µL Stop Solution 2m
4 °C 00:02:00

21 38.7 µL XP RNA Beads (1.8X) 5m
Room temperature 00:05:00

22 Room temperature 00:03:00 magnet 3m
Discard supernatant

23 50 µL 80% EtOH 00:00:30 30s
Discard supernatant

24 go to step #23 x1

25 Undetermined, 00:00:10 , pulse 10s
Discard supernatant

26 Remove from magnet
17 µL NAF
1000 rpm, 20°C, 00:01:00

27 16 µL ds DNA to new 0.2 mL PCR tube

cDNA synthesis (using SMARTscribe reverse transcriptase kit, Clontech).

28 Prepare RT master mix at **room temperature** (22 uL total; 1.1X per sample):
- 8 µL 5X First STrand buffer
- 1.5 µL DTT (20 mM)
- 4 µL dNTP mix (10 mM)
- 2 µL RNAse Inhibitor
- 2 µL isoTSO (12 uM)
- 2 µL SMARTScribe RT
- 2.5 µL NAF

29 In 0.2 mL PCR tube from **step 27**
- 16 µL ds DNA
- 2 µL RT Primer 1 (10 uM); custom ds DNA

30 10s
- 1000 rpm, Room temperature , 00:01:00
- Undetermined, 00:00:10 , pulse

31 1h 13m
- 72 °C 00:03:00
- 42 °C **HOLD** - add 22 µL RT master mix
- 42 °C 01:00:00
- 70 °C 00:10:00
- 4 °C **HOLD**

32 5m
- 24 µL XP DNA Beads (0.6X)
- Room temperature 00:05:00

33
- Room temperature 00:03:00 magnet

Discard supernatant

34
- 50 µL 80% EtOH 00:00:30

Discard supernatant

35
- go to step #23 x1

36 Undetermined, 00:00:10 , pulse
Discard supernatant

37 Remove from magnet
42 µL NAF
1000 rpm, 20°C, 00:01:00

38 40 µL ds DNA to new 0.2 mL PCR tube

cDNA library amplification (using Advantage 2 PCR enzyme system, Clontech).

11m 25s

39 Prepare the cDNA library amplification rxn:
40 µL ds DNA
10 µL 10X Advantage 2SA PCR buffer
2 µL dNTP (10 mM)
2 µL 5' PCR Primer II A (SMARTer PCR kit)
2 µL Univ. RV Primer (Poly(A) Tail Kit)
42 µL NAF
2 µL Advantage 2 Polymerase Mix

40 Perform PCR 11m 25s
1. 98 °C **HOLD** - add cDNA rxn
2. 98 °C 00:01:00
3. 98 °C 00:00:10
4. 63 °C 00:00:15
5. 68 °C 00:03:00 **repeat 40.3 -40.5 24X**
6. 68 °C 00:07:00
7. 4 °C **HOLD**

41 60 µL XP DNA Beads (0.6X)
Room temperature 00:05:00

42 Room temperature 00:03:00 magnet

Discard supernatant

43 200 µL 80% EtOH 00:00:30
Discard supernatant

44 go to step #23 x1

45 Undetermined, 00:00:10 , pulse
Discard supernatant

46 Remove from magnet
42 µL NAF
1000 rpm, 20°C, 00:01:00

47 40 µL ds DNA to new 0.2 mL PCR tube

Sequencing library preparation (using the SMRTbell™ Template Prep Kit)

48 Performed in PacBio sequencing core