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Transposon mapping using flanking sequence exponential anchored (FLEA) PCR

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Abstract

This protocol describes a modified version of flanking sequence exponential anchored (FLEA) PCR. The original FLEA-PCR method was developed to amplify integration sites of retroviruses or retro-transposons. Such transposons contain two long terminal repeats (LTR), which have the same orientation. In contrast, DNA transposons have inverted terminal repeats (ITR), which are inverted in their orientation. Here, we adapt FLEA-PCR for mapping genomic DNA transposition, suitable for the analysis of DNA transposition reporters and genomes engineered through the use of DNA transposition.

Subject terms: Cell biology Genetic analysis Genetic modification Genomics Nucleic acid based molecular biology

Keywords: Transposition genomic integration PCR flanking sequence PCR

Reagents

1) Oligonucleotides generated by solid-phase synthesis and purified by HPLC:

Biotinylated primer: 5’-[BioTEG]CATTTTGACTCACGCGGTCGT-3’

Anchor: 5’-GTGGCACGGACTGATCNNNNNN-3’, where NNNNNN is a random hexamer sequence

Exponential: 5’-GTGGCACGGACTGCA-3’

Transposon1: 5’-ATTGACAAGCACGCCTCACG-3’

Transposon2: 5’-ATGCACAGCGACGGATTCG-3’

2) PureLink Genomic DNA Mini Kit (#K1820-00, Invitrogen, Waltham, MA, USA)

3) Platinum PCR SuperMix High Fidelity (#12532-016, Invitrogen, Waltham, MA, USA)

4) Water, DNase and RNase free (#BP2484-100, Fisher, Waltham, MA USA)

5) Dynabeads kilobaseBINDER Kit (#60101, Invitrogen, Waltham, MA, USA)

6) T7 DNA Polymerase (#M0274S, New England BioLabs, Beverly, MA, USA)

7) Deoxyribose nucleotide triphosphates dNTP (#18427-088, Invitrogen, Waltham, MA, USA)

8) PureLink PCR Purification Kit (#K3100-01, Invitrogen Corp., Carlsbad, CA, USA)

Equipment

1) DynaMag- 96 Well slide magnet (#12331D, Life technologies, Carlsbad, CA, USA)

2) GeneMate UltraFlux I, 8-Strip Standard PCR Tubes (#T-3135-1, Bioexpress, Kaysville, UT, USA)

3) Eppendorf Master cycler ProS (Eppendorf, Hamburg, Germany)

4) Eppendorf Thermomixer R (Eppendorf, Hamburg, Germany)

5) Amicon Ultra 0.5mL 100K (#UFC510096, EMD Millipore, Billerica, MA, USA)

Procedure

Linear Amplification

This step anneals biotinylated primer specific to the mobilized transposon and generates a linearly-extended single strand that spans the genomic junction of the transposon. Sequence of the biotinylated primer is specifically designed to hybridize with a unique sequence of the transposon reporter or transposition-mediated transgene, with the sufficiently long extension time to span the transposition breakpoint. In the case of this protocol designed to identify synthetic piggyBac transposon insertions, the extension time was determined by the 100 nucleotides separating the primer from the DNA transposon ITR (Fig. 1A).

1. Prepare the PCR mixture in GeneMate UltraFlux tubes by mixing:

1 µL of 5 µM Biotinylated primer (100 nM)
4 µL genomic DNA (2 µg)
45 µL Invitrogen Platinum high fidelity SuperMix

2. Carry out annealing and linear extension using the following PCR conditions:
95°C for 5 min
30 cycles of :
95 °C (45 seconds)
62 °C (45 seconds)
72 °C (3 min)
1×72 °C for 10 min
4 °C until next step

3. Transfer PCR reaction products into Amicon Ultra 0.5 100K filter tubes, add 200 µl of nuclease-free water

4. Centrifuge at 12,000 g for 10 minutes at room temperature, 42 µl concentrated purified sample should remain

5. Invert the column in clean tubes and centrifuge at 1,000 g for 2 min

Isolation of Biotinylated Amplicon

1. Transfer 3 µL of Dynal magnetic streptavidin beads into GeneMate UltraFlux tubes

2. Place the tubes onto DynaMag 96 magnetic holder

3. Remove supernatant

4. Add 20 µL kilobaseBINDER kit Binding solution

5. Place tube onto DynaMag 96

6. Remove solution

7. Resuspend magnetic beads in 10 µL Binding solution

8. Add 5 µL of isolated PCR product from the previous step

9. Add 5 µL of nuclease-free water

10. Shake tube at room temperature for 3 hours

Purification of Biotinylated Amplicon

1. Place tube onto DynaMag 96

2. Remove supernatant

3. Add 40µL of kilobaseBINDER kit Washing solution

4. Place tube onto DynaMag 96

5. Remove 40 µL Washing solution

6. Add 40 µL of nuclease-free water

7. Remove 40 µL water

8. Add 20 µL of 0.1M NaOH

9. Shake for 30 min at 37 C

10. Place tube onto DynaMag 96

11. Remove NaOH

12. Add 40 µL nuclease-free water

13. Place tube on DynaMag 96

14. Remove water

Synthesis of Complementary Strand

This step uses a degenerate primer to anneal and synthesize the complementary strand of the biotinylated amplicons. The sequence of the anchor primer was adapted from Pule et al. (1). The primer has a 3’ degenerate sequence and a 5’ adapter sequence that enables the anchor primer to prime to the 3’ end of the linear product of the previous step, containing the unknown genomic sequence flanking the inserted transposon (Fig. 1B).

Prepare the PCR buffer mixture by mixing:
15 µL nuclease-free water
2 µL 10x T7 DNA Polymerase Buffer
2 µL of 100 µM Anchor primer
1 µL of 10 mM dNTP (0.5 mM)

1. Add 19 µL of this mixture to tube with streptavidin beads and mix by pipetting

2. Incubate at 95°C for 1 minute and decrease temperature to 37 °C over 10 minutes

3. Centrifuge to collect any condensate and add 1 µL of T7 DNA polymerase

4. Incubate at 37 °C for 1 hour

At this point a linear double stranded DNA product has been synthesized, which spans the transposon terminal repeat as well as the flanking genomic sequence.

Product Amplification

This step uses exponential PCR to amplify the isolated double-stranded DNA sequences using adapter sequences included in the primers used for linear extension and complementary strand synthesis. As a result of this PCR, an amplicon is produced that includes sequences of the transposon terminal repeats and the flanking genomic sequences (Fig. 1C).

Wash the streptavidin beads to remove remaining anchor primers and carry out PCR:

1. Place tube on DynaMag 96 and remove supernatant

2. Add 40 µL of water

3. Place tube on DynaMag 96 and remove supernatant

4. Repeat 4 times

5. Prepare the PCR mixture:

1 µL of 25 µM Transposon1 primer
1 µL of 25 µM Exponential primer
45 µL of Invitrogen Platinum High Fidelity SuperMix

6. Add 48 µL of PCR mixture to the beads and mix by pipetting

7. Carry out PCR using the following conditions:

95 °C for 5 minutes
35 cycles of:
95 °C for 45seconds

62 °C for 45seconds
72°C for 3 minutes
1×72 °C for 10 minutes
4 °C until next step

Nested Product Amplification

In order to increase the yield of specific amplicons spanning the breakpoint between transposon and flanking human genome, a nested PCR can be used (Fig. 1D).

1. Use PureLink PCR Purification Kit to purify PCR product amplicons. Elute in 50 µL nuclease-free water

2. Prepare PCR reaction mixture:
1 µl of 25 µM Transposon 2 primer
1 µl of 25µM Exponential primer
45 µl Invitrogen Platinum High Fidelity SuperMix

3. Add 3 µl of purified PCR product from previous PCR amplification to 47 µL of PCR mixture in GeneMate UltraFlux tubes

4. Carry out PCR using the following conditions:

95 °C for 5 minutes
35 cycles of:
95 °C for 45seconds
62 °C for 45seconds
72°C for 3 minutes
1×72 °C for 10 minutes
4 °C until next step

5. Purify PCR product and proceed with cloning for capillary Sanger DNA sequencing or library preparation for massively parallel single molecule DNA sequencing.

Timing

The entire procedure can be completed in two days.

References

1. Pule MA, et al. (2008) Flanking-sequence exponential anchored-polymerase chain reaction amplification: a sensitive and highly specific method for detecting retroviral integrant-host-junction

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2. Fraser MJ, Cary L, Boonvisudhi K, & Wang HG (1995) Assay for movement of Lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. Virology 211(2):397-407.

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Acknowledgements

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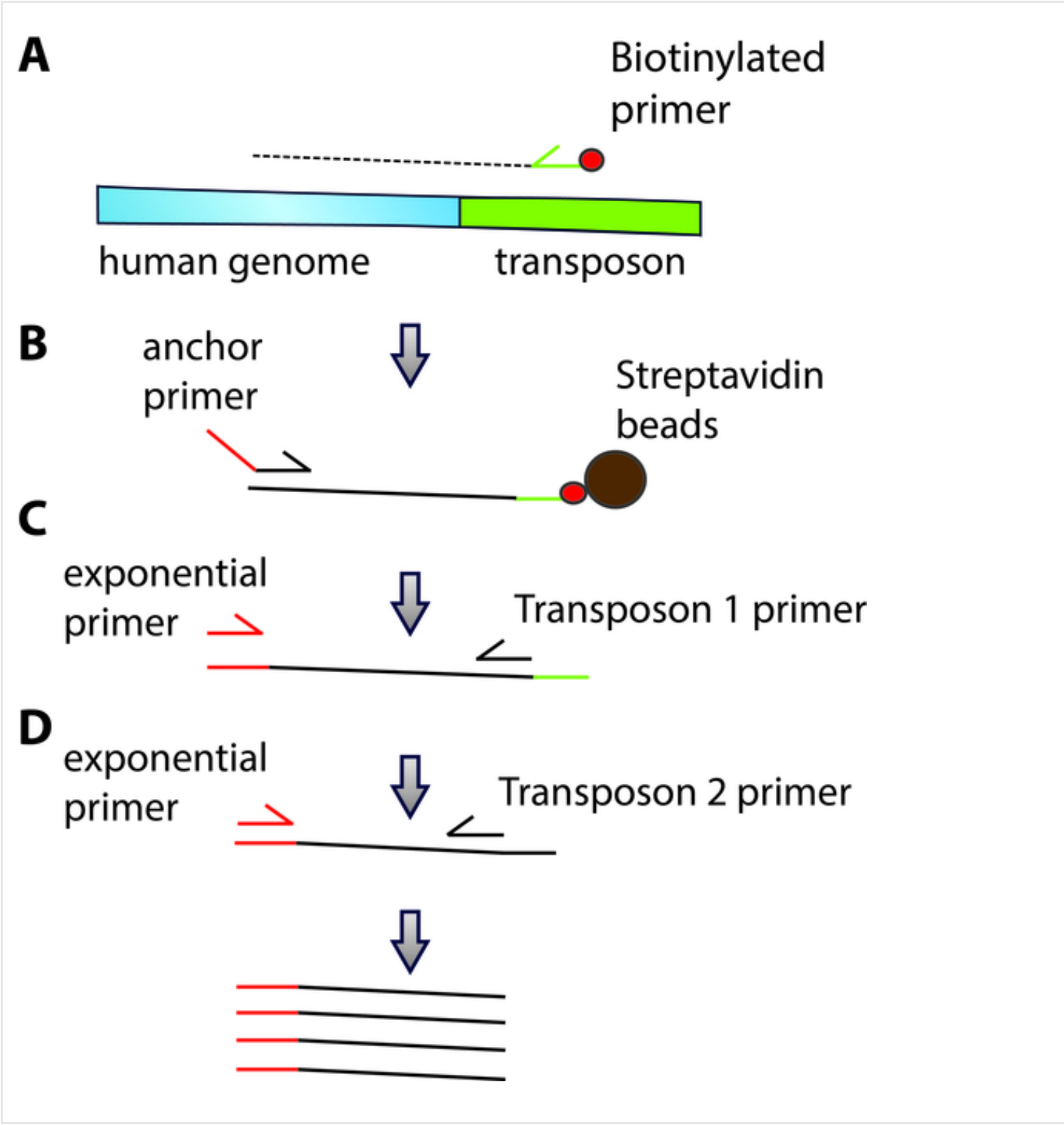
Figures

Figure 1: Schematic of transposon specific flanking-sequence exponential anchored–polymerase chain reaction amplification (FLEA-PCR) assay for amplifying genomic DNA transposon insertions.



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Competing financial interests

None.

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