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Single-cell analysis of functional heterogeneity in DNA repair capacity

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ABSTRACT

Methods to measure heterogeneity among cells are rapidly transforming our understanding of biology but are currently limited to static measurements of DNA, RNA, and protein abundance. We developed an approach to simultaneously measure biochemical activities and mRNA abundance in single cells to understand the heterogeneity of DNA repair across thousands of human lymphocytes, identifying known and novel cell-type-specific DNA repair phenotypes. Our method provides a general framework for understanding functional heterogeneity among single cells.

MATERIALS

NAME	CATALOG #	VENDOR
NEBNext End Repair Module - 100 rxns	E6050L	New England Biolabs
Nuclease-free Water		
Fresh 80% Ethanol		
Thermal cycler		
Chromium Single Cell 3' Library & Gel Bead Kit v2	120267	10x Genomics
AmpureXP beads	A63880	Beckman Coulter
polyA DNA repair substrates		
Klenow (3'→5' exo-)	P7010-LC-L	Enzymatics
10X Blue Buffer	B0110	Enzymatics
Y adapter (see step 8)		
T4 DNA ligase (Rapid) 600000 U/mL	L6030-HC-L	Enzymatics
2X Rapid Ligation Buffer	B1010	Enzymatics
ST Buffer (10 mM Tris 7.5 50 mM NaCl)		
PCR primers (see step 9)		
Phusion		
5x Phusion HF buffer		
10 mM dATP		
10 mM dNTPs		
0.2 mL PCR Strip Magnetic Separator		
10% 29:1 acrylamide TBE gel		
D1000 Screen Tape		Agilent Technologies

Overview

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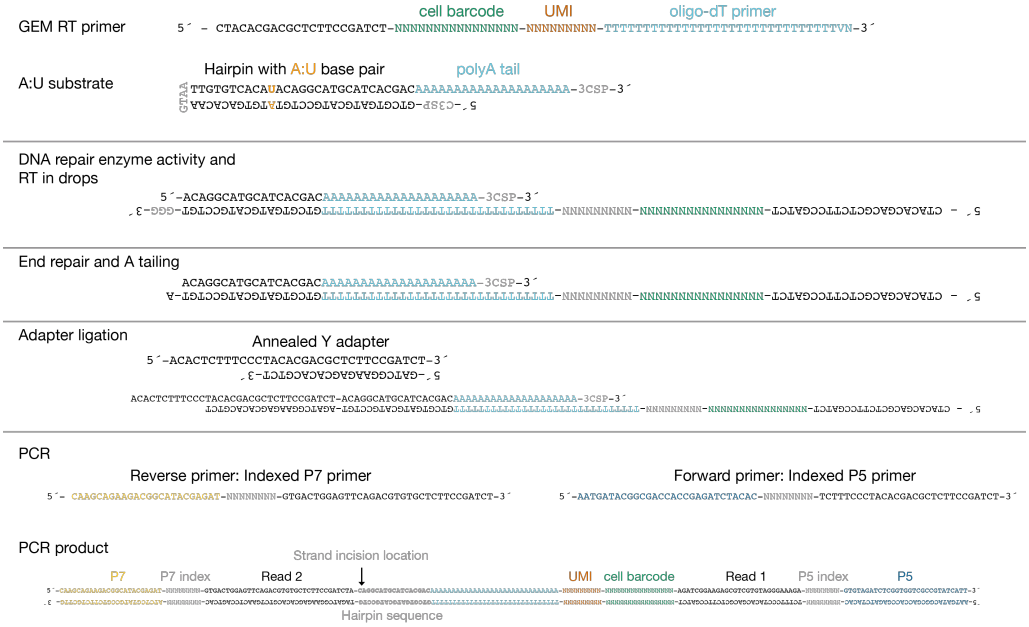
Below is an overview of the repair library preparation.



PolyA hairpin substrate design

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DNA repair substrates were designed as DNA hairpins containing a single modified base. The hairpin has a 20 nucleotide polyA single stranded region that enables hybridization to oligo-dT primers in the 10X GEMs.

Substrate	Sequence
A:U - 1	/5SpC3/GTCGTGATGCATGCCTGTATGTGACACAAGTAATTGTGTCACAUACAGGCATGCATCACGAC AAAAAAAAAAAAAAAAAAAA/3SpC3/
C:riboG - 1	/5SpC3/ACTCGAGTCACACTCGTACTGATGCATGAGTAATCATGCATCArGTACGAGTGTGACTCGAGT AAAAAAAAAAAAAAAAAAAA/3SpC3/
G:U	/5SpC3/TGAATTCGAGAGTCGTTCGGCGATATAACGTAAGTTATATCGCUGAACGACTCTCGAATTCA AAAAAAAAAAAAAAAAAAAA/3SpC3/
G:Abasic	/5SpC3/ACGTACGTTAGCATAACTGTAATCTTAATGTAAATTAAGATTA/idSp/AGTTATGCTAACGTAC GTAAAAAAAAAAAAAAAAAAAA/3SpC3/
C:I	/5SpC3/GAGCGCTACTCAGATGACTTCGAGTGATTGTAAAATCACTCGAIGTCATCTGAGTAGCGCTCA AAAAAAAAAAAAAAAAAAAA/3SpC3/
T:I	/5SpC3/AGTGCACGCTCTATGTATCGAAGAGTTGTGTAAACAACTCTTCIATACATAGAGCGTGCACTA AAAAAAAAAAAAAAAAAAAA/3SpC3/
Normal	/5SpC3/CGCTAGCCTTCAGCTATCTTCTACCCATCGTAAGATGGGTAGAAGATAGCTGAAGGCTAGCG AAAAAAAAAAAAAAAAAAAA/3SpC3/
A:U - 2	/5C3Sp/GCTTGCCTTGTCGATCACAAGTATGTCAGGTAACTGACATACTUGTGATCGACAAGGCAAGC AAAAAAAAAAAAAAAAAAAA/3C3Sp/
A:U - 3	/5C3Sp/GCTGGCCTTTGCACTAGGAACTTACCGCGGTAACGCGGTAAGTUCCTAGTGCAAAGGCCAGC AAAAAAAAAAAAAAAAAAAA/3C3Sp/
A:U - 4	/5C3Sp/TGCCAACGGTGGAGTACGAGGTAAGAAGCGTAAGCTTCTTACCUCGTACTCCACCGTTGGCA AAAAAAAAAAAAAAAAAAAA/3C3Sp/
A:U - 5	/5C3Sp/ATGGTTCACGTGGGACATAGCGATCGTGCGTAAGCACGATCGCUATGTCCCACGTGAACCAT AAAAAAAAAAAAAAAAAAAA/3C3Sp/
C:riboG - 2	/5C3Sp/TCCGACGGCAAGAGTCCTCTCCAATTACCGTAAGGTAATTGGArGAGGACTCTTGCCGTCGGA AAAAAAAAAAAAAAAAAAAA/3C3Sp/
C:riboG - 3	/5C3Sp/TCAATTGTTGGCAGAGGCCAATTAGTGTCGTAAGACACTAATTrGGCCTCTGCCAACAATTGA AAAAAAAAAAAAAAAAAAAA/3C3Sp/
C:riboG - 4	/5C3Sp/TCGGACCAAGTTATGGGCCGCGAATTTCCGTAAGGAAATTCGCrGGCCCATAACTTGGTCCGA AAAAAAAAAAAAAAAAAAAA/3C3Sp/
C:riboG - 5	/5C3Sp/CTCAGACGAACGTTGCTACGGACCCGTATGTAAATACGGGTCCrGTAGCAACGTTCGTCTGAG AAAAAAAAAAAAAAAAAAAA/3C3Sp/

Substrates were mixed and diluted to a 20x solution (200 nM - 2 µM) in nuclease-free water.

To get robust single cell measurements, substrates should be at least 100 nM each in 20x solution, however, we measured repair with substrates as low as 20 nM in 20x solution.

10x Kit Changes - GEM generation

3 Follow the 10x protocol (CG00052 Rev F / CG000075 Rev C) for GEM generation with the following changes:

1. Using the cell suspension volume calculator table, subtract 5 µl from the Volume of Nuclease-free Water.
2. Add 5 µl of 20x hairpin solution per sample to the MM after you have added nuclease-free water and before the cells are added to the MM (i.e., between steps 1.1C and 1.1D).

CRITICAL: You must add DNA repair substrates (polyadenylated hairpins) to nuclease-free water added to master mix.

10x Kit Changes - RT

4 Collect GEMs from chip and proceed directly to GEM-RT incubation below.

CRITICAL: Do not follow the RT protocol from 10x protocols.

Lid Temperature	Reaction Volume	Run Time
53 ˚C	125 µl	~2 hours
Step	Temperature	Time
1	37 ˚C	60 min
2	53 ˚C	45 min
3	4 ˚C	Hold

Since this protocol does not denature the RT enzyme, proceed directly to Post GEM-RT Cleanup.

10x Kit Changes - Post GEM-RT Cleanup and cDNA amplification

5 Follow 10x protocol for Post GEM-RT Cleanup - Silane Dynabeads protocol (section 2.1)

After elution from silane beads in 35 µl, perform an SPRIselect size selection:

1. Vortex the SPRIselect Reagent (or Ampure XP) until fully resuspended.
2. Add 0.6x volume of SPRIselect Reagent (21 µl) and pipette to mix.
3. Incubate at room temperature for 5 min.
4. Place on magnetic strip and wait until liquid is clear.
5. **DO NOT DISCARD SUPERNATANT**. Transfer supernatant to new tube. This fraction will be referred to as the **repair substrate fraction**. The beads contain the **mRNA fraction**.
6. Add 41 µl of SPRIselect Reagent (or Ampure XP) to the **repair substrate fraction** (**1.8x**) and pipette to mix.
7. Incubate at room temperature for 5 min.
8. While repair substrate fraction is incubating, add 150 µl 80% EtOH to the **mRNA fraction** while beads are still on magnet.
9. Remove the 80% EtOH and repeat wash (step 8) for a total of 2 washes.
10. Briefly spin down tubes and place back on magnet and remove remaining EtOH.
11. Dry beads for 2 min.
12. Add 35.5 µl EB buffer (from 10x reagents) and incubate for 2 min at room temperature.
13. Place on magnet. Once liquid is clear, remove 35 µl and place into new tube - this is the **mRNA fraction** and will proceed to cDNA amplification step of the 10x protocol.
14. After the 5 min incubate with 1.8X SPRIselect reagent, place the **repair substrate fraction** on the magnet
15. Once the liquid is clear, remove and discard the supernatant.
16. Add 100 µl 80% EtOH to the beads while the beads are still on the magnet.
17. Remove the 80% EtOH and repeat wash (step 15) for a total of 2 washes.
18. Briefly spin down tubes and place back on magnet and remove remaining EtOH.
19. Dry beads for 2 min.
20. Add 20.5 µl of nuclease-free water and incubate for 2 min at room temperature.
21. Place on magnet and once liquid is clear, remove 20 µl and place into new tube - this is the **repair substrate fraction** and will proceed to repair substrate library prep.

10x kit - mRNA library

6 Follow the remaining 10x protocol to finish mRNA library.

Repair library - End Repair

7 Mix the end repair mix on ice (End repair kit from NEB)

Water	6 µl
10x End repair buffer (NEB)	3 µl
End repair enzyme mix (NEB)	1 µl

Add the end repair mix to the 20 µl of **repair substrate fraction** isolated above.
Incubate at 20 °C for 30 minutes.

Clean up:

1. Transfer end repair reaction to 1.5 mL eppendorf tube .
2. Add 130 µl of 0.4 M sodium acetate to the end repair reaction.
3. Add 400 µl 100% EtOH to the sodium acetate and end repair reaction mix.
4. Add 1 µl of glyco-blue coprecipitate (optional).
5. Vortex for 5 seconds to mix.
6. Incubate at -20 °C for 30 minutes.
7. Centrifuge precipitation at >10000 g for 10 minutes at 4 °C.
8. Remove supernatant and add 500 µl 80% EtOH.
9. Centrifuge again at >10000 g for 10 min at 4 °C.
10. Remove supernatant carefully to not disturb the pellet.
11. Let pellet dry for 5 min.
12. Resuspend pellet in 20 µl nuclease free H2O.

Repair library - A-tailing

8 Mix the following:

Water	2 µl
10x Blue buffer (enzymatics)	2 µl
10 mM dATP	2 µl
Klenow exo- (enzymatics)	1 µl
End-repaired repair substrate fraction (from previous step)	13 µl

Incubate at 37 °C for 30 minutes.

1. Transfer A-tailing reaction to 1.5 mL eppendorf tube.
2. Add 130 µl of 0.4 M sodium acetate.
3. Add 400 µl 100% EtOH.
4. If concerned about seeing a pellet, add 1 µl glyco-blue coprecipitate.
5. Vortex for 5 seconds to mix.
6. Incubate at -20 for 30 minutes.
7. Centrifuge precipitation at >10000 g for 10 minutes at 4 °C.
8. Remove supernatant and add 500 µl 80% EtOH.
9. Centrifuge again at >10000 g for 10 min at 4 °C.
10. Remove supernatant carefully to not disturb the pellet.
11. Let pellet dry for 5 min.
12. Resuspend pellet in 20 µl nuclease free water.

Repair library - Adapter ligation

9 Anneal adapters:

Name	Sequence
Y adapter 1	TCTGCACACGAGAAGGCTAG
Y adapter 2	ACACTCTTTCCCTACACGAC GCTCTTCCGATCT

Y adapter sequences

1. Mix 20 µl of each 100 µM adapter piece in 10 mM Tris pH 7.5, 50 mM NaCl (ST buffer).
2. Heat to 95 °C for 5 minutes then cool at -0.1 C/sec to 4 °C.
3. Add 160 µL of cold ST buffer to make 10 µM adapters.
4. Make 10 µL aliquots and freeze at -20 °C until ready to use.
5. When ready to use, thaw on ice. Do not freeze-thaw adapter aliquots.

Reaction mix:

2x ligation buffer (enzymatics)	15 µl
Pre-annealed adapters - 10 µM	1 µl
T4 DNA ligase (Rapid) (enzymatics)	1 µl
A-tailed repair substrate fraction (from previous step)	13 µl

Incubate at 20 °C for 30 min.

Ampure clean up (1.8x)

1. Add 36 µl (1.8x) Ampure XP beads to ligation reaction.
2. Incubate at room temperature for 5 minutes.
3. Place on magnet.
4. Once liquid is clear, remove and discard supernatant.
5. Wash 2x with 150 µl 80% EtOH with beads on magnet.
6. Dry beads for 2 min.
7. Resuspend beads in 20 µl H2O and incubate for 2 minutes at room temperature.
8. Place beads on magnet and once liquid is clear, transfer supernatant to another tube.

Repair library - ILMN PCR

10 Set up master mix:

Indexed primers

Name	Sequence
Forward indexed P5 primer	CAAGCAGAAGACGGCATACGAGA TNNNNNNNNGTGACTGGAGTTC AGACGTGTGCTCTTCCGA*T*C*T
Reverse indexed P7 primer	AATGATACGGCGACCACCGAGAT CTACACNNNNNNNNTCTTTCCCT ACACGACGCTCTTCCGA*T*C*T

N's indicate sample 8-base sample index sequence. * represent phosphorothioate linkage.

Mix 10 µl of 100 µM forward and reverse primers and dilute to 100 µl to make 10 µM primers.

Water	21 µl
5x Phusion buffer HF (NEB)	10 µl
10 mM dNTPs	2 µl
Phusion (NEB)	1 µl

1. Add 34 µl master mix to PCR tube.
2. Add 3 µl 10 µM mixed indexed ILMN primers to tubes, make sure each sample has a unique index.
3. Add 13 µl repair substrate fraction from adapter ligation reaction to tube.

Thermal cycler:

Temperature	Time	
98 °C	3 min	
98 °C	15 sec	14-20 cycles
65 °C	15 sec	
72 °C	15 sec	
72 °C	5 min	
4 °C	Hold	

Repair library - Clean up

11 Run 1 µl of PCR product on 10% 29:1 acrylamide TBE gel or on Agilent D1000 tapesation tape.

Library size should about 200-250 bp.

If there doesn't appear to be a library, add more Phusion to PCR reaction and do 2-5 more cycles of PCR.

Ampure cleanup

1. Add 1x AmpureXP beads to PCR reaction (49 µL).
2. Incubate at room temperature for 5 minutes.
3. Place on magnet and once liquid is clear, remove and discard supernatant.
4. Wash 2x with 200 µl 80% EtOH.
5. Air dry for 2 minutes.
6. Resuspend beads in 20.5 µl H20.
7. Incubate at room temperature for 2 minutes.
8. Place on magnet, and once liquid is clear transfer 20 µl to a new tube.

Sequence at least 28x100 to get all cell barcode and DNA repair information from library.