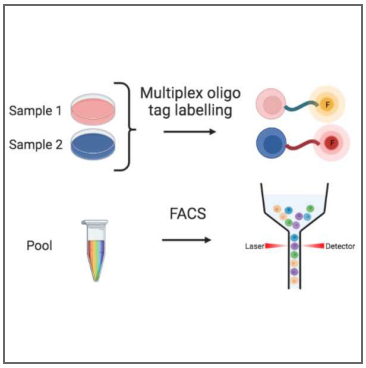

FEB 05, 2024

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.81wgbyjpovpk/v1

Protocol Citation: Daniel V Brown 2024. Optimising sample multiplexing oligos by flow cytometry. **protocols.io** https://dx.doi.org/10.17504/protocols.io.81wgbyjpovpk/v1

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

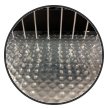
Created: May 14, 2023

Optimising sample multiplexing oligos by flow cytometry

Daniel V Brown[1]

[1]Walter and Eliza Hall Institute

WEHI Advanced Genomics Facility



Daniel V Brown
Walter and Eliza Hall Institute

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ABSTRACT

Optmisation of sample multiplexing oligos by scRNA-Seq is costly and time consuming. A cheaper and faster method is to use a flow cytometry read-out with fluorescent detection oligonucleotides.
This method can also be used to mix samples with different fluorescently labelled oligos and investigate signal swapping.

IMAGE ATTRIBUTION

Made with Biorender.com

Last Modified: Feb 05, 2024

PROTOCOL integer ID: 81857

Keywords: scRNA-Seq, facs, sample multiplexing, hashtag

GUIDELINES

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MATERIALS

- Phosphate Buffered Saline, without magnesium and calcium
- Sample multiplexing oligos (MULTI-Seq, CellPlex or Hashtag Antibody)
- 10ug/mL DAPI
- 30% BSA stock solution
- Fluorescent detection oligonucleotides

	A	B	C
	Name	Sequence	Modification
	A647_FB2_detect	/5Alex647N/CCTTAGCCGCTAATAGGTGAGC	5' Alexa 647 modification
	A594_FB2_detect	/5Alex594N/TTGCTAGGACCGGCCTTAAAGC	5' Alexa 594 modification
	A594_FB1_detect	/5Alex594N/TTGCTAGGACCGGCCTTAAAGC	5' Alexa 594 modification
	A647_Total-SeqC_detect	/5Alex647N/CTGTCTCTTATACACATCTCCG	5' Alexa 647 modification
	AF647_oligo_dT_detect	/5Alex647N/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	5' Alexa 647 modification

Fluorescent detection oligonucleotides. Order with HPLC modification

MULTI-Seq barcoding oligos. I substituted the poly-A tail for 10x Genomics feature barcode 2 sequence.

	A	B	C
	Name	Sequence	Barcode
	multiSeq_FB2_BC2	CCTTGGCACCCGAGAATTCCACCACAATGGCTCACCTATTAGCGGCTAAGG	CCACAATG
	multiSeq_FB2_BC3	CCTTGGCACCCGAGAATTCCATGAGACCTGCTCACCTATTAGCGGCTAAGG	TGAGACCT
	multiSeq_FB2_BC4	CCTTGGCACCCGAGAATTCCAGCACACGCGCTCACCTATTAGCGGCTAAGG	GCACACGC
	multiSeq_FB2_BC5	CCTTGGCACCCGAGAATTCCAAGAGAGAGGCTCACCTATTAGCGGCTAAGG	AGAGAGAG
	multiSeq_FB2_BC6	CCTTGGCACCCGAGAATTCCATCACAGCAGCTCACCTATTAGCGGCTAAGG	TCACAGCA
	multiSeq_FB2_BC7	CCTTGGCACCCGAGAATTCCAGAAAAGGGGCTCACCTATTAG	GAAAAGGG

	A	B	C
		CGGCTAAGG	
	multiSeq_FB2_BC 8	CCTTGGCACCCGAGAATTCCA CGAGATTCGCTCACCTATTAG CGGCTAAGG	CGAGAT TC
	multiSeq_FB2_BC 9	CCTTGGCACCCGAGAATTCCA GTAGCACTGCTCACCTATTAG CGGCTAAGG	GTAGCA CT
	multiSeq_FB2_BC 10	CCTTGGCACCCGAGAATTCCA CGACCAGCGCTCACCTATTAG CGGCTAAGG	CGACC AGC
	multiSeq_FB2_BC 11	CCTTGGCACCCGAGAATTCCA TTAGCCAGGCTCACCTATTAG CGGCTAAGG	TTAGCC AG
	multiSeq_FB2_BC 12	CCTTGGCACCCGAGAATTCCA GGACCCCAGCTCACCTATTAG CGGCTAAGG	GGACC CCA
	multiSeq_FB2_BC 13	CCTTGGCACCCGAGAATTCCA CCAACCGGGCTCACCTATTAG CGGCTAAGG	CCAACC GG

SAFETY WARNINGS

Please follow all Manufacturer safety warnings and recommendations.

Prepare multiplexing reagent

1 For CellPlex and Hashtag antibody the reagent comes ready to use.

1.1 Prepare a dilution series for titration if desired

MULTI-Seq oligo preparation

2 Mix anchor and barcode strands in 1:1 molar ratio in PBS (without FBS or BSA at 2 μM concentration (10X stock).

2.1 This is 6uL 50uM anchor LMO, and 15uL 10uM barcode oligo to 129uL plain PBS.

2.2 Make one unique barcode solution per sample.

2.3 Total 25 µL per sample.

3 Make a 10X solution of the Co-Anchor in PBS.

3.1 Add 3uL 50uM co-anchor to 141uL plain PBS.

3.2 Add 6uL 100uM fluorescent detection oligo. For example:
Alexa 647 feature barcode 2 detection oligo.

3.3 It is highly recomended to label in at least duplicate. On these occasions I label the other replicate with a Alexa 594 detection oligo and mix immediately before FACS analysis.

Sample preparation

4 I use suspension cell lines to titrate cell multiplexing oligos so the sample preparation is easy.

4.1 Prepare a single-cell suspension of the sample to be tested.

4.2 Wash cells once in plain PBS without additives. I centrifuge suspension cell lines at 400xg. Resuspend in PBS.

4.3 Count cells and transfer 100k to 1M cells (preferably 500k) into a 1.5mL tube for labelling

Labelling samples with multiplexing oligos

5 This is largely based on original protocols but with the addition of a fluorescent secondary oligo.

5.1 Resuspend cells in 180uL of plain PBS.

5.2 Add 20 µL 10X Anchor:Barcode solution and pipette gently to mix 10 – 15 times.

5.3 Incubate on ice for 5 minutes.

5.4 Add 20 µL Co-Anchor solution and pipette gently to mix.

5.5 Incubate 5 minutes longer on ice.

5.6 Add 1.5mL of 1% BSA in PBS (ice cold) to quench.

5.7 Add 1.5mL of 1% BSA in PBS (ice cold) to quench.

5.8 Add 1mL of 1% BSA in PBS (ice cold) to quench.

5.9 Centrifuge cells at 4°C 400xg 5min.

5.10 Resuspend cells in the remaining 100uL of supernatant then transfer to a new 1.5mL tube.

5.11 Add 1.9mL 1% BSA in PBS and spin 5 minutes 400g at 4°C.

5.12 Repeat wash 2 more times for a total of 3 washes.

5.13 Resuspend cells in 500 µL of PBS + 1% BSA and transfer to 5mL polystyrene FACS tube

Flow cytometry

6 Use a FACS analyser to compare the signal of labelled samples to a control sample where only the fluorescent detection oligo has been added.
If you have replicate labelled samples with different fluorescent detection oligos, you may combine them at this step.

6.1 Add DAPI to a final concentration of 0.1ug/mL (1/100 stock tube).

6.2 Gate for debris, single cells and viable cells.

6.3 Aquire at least 10,000 viable cells per test.

6.4 Analyse with relevant software.