WILDseq Library Prep v2 Protocol

Callum Malcolm

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Contents

Reverse Transcription

Overview

- cDNA synthesis from RNA template
- Include a -RT control if possible
  - Mix several samples and include extra H20 instead of Reverse Transcriptase
  - PCR this sample as well, checks for contamination of genomic DNA, mastermix, ect.
  - Can also use as negative control to determine product size and yield on tapestation

Materials

- For 40 samples

Component	Expected Volume/experiment	Material ID
5x SSIV Buffer	200 μL	
SSIV RT	50 μL	
100 mM DTT	50 μL	
RNAse Out	50 μL	
Thermolabile Exonuclease I	1 μL	
NEBuffer r3.1*	2 μL	

RT Protocol

1. In PCR strip tubes, prepare 1-5 μg of RNA in a total volume of 10 μl of RNAse/DNAase-free water.
2. Add 1 μl of WS_RT_UMI_NexteraR2 primer (2μM stock)
3. Add 1 μl dNTPs (10 mM each).
4. Denature at 65 C for 5 mins in the PCR machine, then straight onto ice for at least 2 mins, spin briefly to get liquid to the bottom of the tube.
5. Prepare mastermix of RT enzyme and buffers. 1.1x the number of samples.

Component	Volume	MM volume
5x SSIV Buffer	4 μL	
SSIV RT	1 μL	
100 mM DTT	1 μL	
RNAse Out	1 μL	

6. Add 7 μl of RT MM prepared above to each sample and mix, spin briefly to get liquid to the bottom of the tube.
   - Spin briefly to get liquid to bottom of the tube
7. In the PCR machine incubate at 53 C for 10 mins followed by 80 C for 10 mins.
8. Add 1 μl Themolabile Exonuclease I (NEB M0568) to remove excess RT primer.

Component	Volume	MM volume
Thermolabile Exonuclease I	1 μL	
NEBuffer r3.1*	2 μL	

Most PCR buffers are compatible

9. In PCR Machine: heat at 37C for 4 mins followed by 80C for 1 min
   - Spin briefly to get liquid to bottom of the tube
10. Add 1 μl of RNAse H and incubate at 37 C for 20 mins.
11. Dilute final cDNA 1:2 with DNAse/RNAse-free water.

PCR Step 1

Overview

- Aim to keep number of cycles at a minimum.
- Forward primer is an equal mix of primers with different length spacers to generate diversity required for sequencing.

Materials

Component	Expected Volume/experiment	Material ID
10uM WS_RT-PCR1_Fwd	70 μL	-
10uM WS_RT-PCR1_Rev	70 μL	-
DNAse/RNAse H20	550 μL	
Kapa Hifi HotStart Ready Mix (2X)	1200 μL	KK2601

PCR1 Protocol

- Set up PCR reactions on ice
- One reaction per sample plus -RT control

1. Prepare mastermix of below reagents at 1.1x the number of samples

Component	Volume
10uM WS_RT-PCR1_Fwd	1.5 μL
10uM WS_RT-PCR1_Rev	1.5 μL
DNAse/RNAse H20	12 μL
Kapa Hifi HotStart Ready Mix (2X)	25 μL
Total Master Mix volume	**40 μL**
cDNA from above	10 μL

2. Perform PCR using the following parameters:

Step Name	Steps	Time
Initial Denaturation	Step 1: 95C	3mins
Denaturation	**Step 2: 98C**	**20s**
Annealing	**Step 3: 60C**	**15s**
Extension	**Step 4: 72C**	**15s**
Final Extension	Step 5: 72C	1 min
Hold Step	Step 6: 12C	Hold

10-25 cycles of steps 2-4

Clean up reaction using Ampure beads and check product size and yield on tapestation

Bead Clean Up

Overview

- Clean PCR reaction by removing leftover primers
- This prevents false reads, cleaner indexing in later steps, and higher quality samples

Materials

- For 40 samples

Component	Expected Volume/experiment	Material ID
Ampure Bead Kit	200 μL	

Protocol

1. Equilibrate Ampure beads to room temp (available from supplies cold room)
2. Mix well and vortex for 30sec to ensure uniform distribution.
3. Add 90ul (1.8X volume) to PCR reaction and mix thoroughly by pipetting.
4. Incubate 5min, room temp
5. Place on magnet for 2 min and keep on magnet until final elution
6. Remove supernatant and add 200ul 70% ethanol (make fresh)
7. Incubate 30sec and remove
8. Repeat 70% ethanol wash
9. Air dry beads 5-10min room temp. Once dry the beads will go from shiny to matt in appearance. Avoid over drying (cracked appearance).
10. Add 15 ul EB buffer and resuspend beads by pipetting
11. Incubate 2min room temp
12. Place on magnet and remove eluate
13. Assess PCR product size, contamination and concentration on tapestation.
    Expected size = 165-172bp

PCR Step 2

Overview

- PCR to index samples for sequencing by attaching UMI
- VERY IMPORTANT to accurately record which samples receive which adaptors
  *Guide for Index Adaptor Pooling

Materials

Component	Expected Volume/experiment	Material ID
10uM Nxxx Nextera i7 adapter	-	-
10uM Sxxx Nextera i5 adapter	-	-
DNAse/RNAse H20	1000 μL	
Kapa Hifi HotStart Ready Mix (2X)	1200 μL	KK2601

PCR2 Protocol

1. Prepare samples according to the following table:

- Keep careful note of which adaptors are added to which sample

Component	Volume
10uM Nxxx Nextera i7 adapter	1.5 μL
10uM Sxxx Nextera i5 adapter	1.5 μL
DNAse/RNAse H20	21 μL
Kapa Hifi HotStart Ready Mix (2X)	25 μL
10ng/μL PCR1	1 μL

2. Perform PCR using the following parameters:

Step Name	Steps	Time
Initial Denaturation	Step 1: 95C	3mins
Denaturation	**Step 2: 98C**	**20s**
Annealing	**Step 3: 55C**	**15s**
Extension	**Step 4: 72C**	**15s**
Final Extension	Step 5: 72C	2 min
Hold Step	Step 6: 12C	Hold

8 cycles of steps 2-4

3. Perform Ampure bead clean up as above with 90ul beads per 50ul PCR reaction.

- If you don't have a crazy number of samples you might also want to consider doing up to 4 PCR2 reactions per sample and pooling them afterwards just to make sure all your sequences are captured.

4. Check size and concentration on tapestation. Dilute and pool samples for sequencing.