Supplemental protocol for **FFPEcap-seq: a method for sequencing capped RNAs in formalin-fixed paraffin-embedded samples**

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FFPE Cap Seq is a protocol designed to allow for the sequencing of 5' ends from highly degraded RNA. Once RNA is availed, the entire protocol can easily be completed in a single work day.

Step 1. Digestion of degraded RNA and rRNA.

In this step we remove RNAs with 5' hydroxyls and phosphates. This includes most rRNAs and RNA degradation products. We use polynucleotide kinase to phosphorylate 5' hydroxyls, and terminator nuclease, a processive 5' to 3' phosphate dependent exonuclease to degrade these products. Transcripts with a 5' cap are protected and thus enriched by this step. We typically see a 70 -95 % reduction in RNA at this step.

Table 1

RNA Digest	
Reagent	**1x**
Buffer A	2
ATP	2
PNK	2
Terminator	1
Super RNase-in	1
H_20	12 – X
RNA	X
Total	20 µL

Each sample is incubated at 30^{o}C for 1 hour, then purified using zymogen RNA clean and concentrate. Sample is eluted in 5 µL of water. 1 µL is used to quantify the remaining RNA and confirm digestion while the other 4 µL are used in the downstream reaction.

Step 2. Reverse Transcription/ Template Switching

Trehalose-sorbitol solution was made by adding 5 mL of a saturated trehalose solution (Sigma) to 10 mL of 1.28 g/mL sorbitol solution (Sigma). 1 g of celex 100 (Sigma) was added to the solution and vortexed vigorously and allowed to sit for 3 hours at room temperature. The tube was then centrifuged and the supernatant was aliquoted and stored at -30°C. Mix the Reverse Transcription (RT) primer (100 µM) and the template switching (TS) oligo (1mM) with trehalose-sorbitol solution. 1 µL of this primer mix is then combined with the RNA and incubated to unfold the RNA. This RNA/Primer solution is then mixed with the Reverse Transcription master mix to perform reverse transcription.

Table 2

Primer Mix

Trehalose Sorbitol Solution	8
RT primer (100 µM)	1
Template Switching Oligo (1mM)	1

*This primer mix is sufficient for 10 reactions

Trehalose-Sorbitol solution was mixed with the template switching and reverser transcription oligo and mixed thoroughly by pipetting up and down 20 times.

Table 3

Pre-Incubation	
Reagent	**1x**
Primer Mix	1 µL
RNA	4 µL

RNA and 1 µL of the primer mix were mixed and incubated for 10 minutes at 65^{o}C, then placed on ice for at least 2 minutes (.

Table 4

RT Master Mix	
Reagent	**1x**
10 mM dNTPs	0.625 µL
5x RT buff	2 µL
5M betaine	1.5 µL
200 mM DTT	0.5 µL
RT	1 µL
Total	5.625 µL

RNA/primer mix and the Reverse transcription master mix were mixed together by pipetting (table 5).

Table 5

Reverse Transcription	
Reagent	**1x**
RNA/primer Mix	5 µL
RT Master Mix	5.625 µL
Total	10.625 µL

Samples were mixed with pipetting and then placed into the thermocycler (table 6).

Table 6

Temperature (^{o}C)	**Duration**	**Cycles**
22	10 minutes	1
40	30 minutes	

72	15 minutes
4	2 minutes

Samples were purified using AMPure XP at a 1:1.8 sample to bead ratio following stand AMPure protocol and eluted in 40 µL of water.

Step 3. 2nd Strand Synthesis and Amplification

During this step 7.5 µL of cDNA is amplified using PCR (Table 7).

Table 7

2nd Strand PCR	
Reagent	**1x**
Phusion MM	25 µL
10 µM Primer F	2.5 µL
10 µM Primer R	2.5 µL
cDNA	7.5 µL
H20	12.5 µL
Total	50 µL

Samples were mixed by pipetting and placed in the thermocycler (Table 8).

Table 8

Temperature (°C)	Duration	Cycles
95	1 minutes	1
95	15 seconds	24
65	10 seconds	
68	2 minutes	
4	hold	1

Samples were purified using AMPure XP at a 1:0.8 sample to bead ratio following standard AMPure protocol and eluted into 30 µL of water.

Step 4. Final library PCR

In this final step we add on the sequence necessary for the cDNA to bind to the Illumina sequencing lane. We quantify the products of the second strand synthesis reaction and add 40 ng of cDNA to the final library PCR reaction (Table 9).

Table 9

Library PCR	
Reagent	**1x**

Phusion MM	25 µL
10 uM Primer F	1.25 µL
10 uM Primer R	1.25 µL
DNA	(40 ng of DNA)
H20	22.5 µL -DNA
Total	50 µL

Samples were mixed by pipetting and placed in the thermocycler (Table 10).

Table 10

Temperature (°C)	Duration	Cycles
95	1 minutes	1
95	15 seconds	
55	10 seconds	2
68	2 minutes	
95	15 seconds	
65	10 seconds	9
68	2 minutes	
4	hold	1

Samples were purified using AMPure XP at a 1:0.8 sample to bead ratio following standard AMPure protocol and eluted into 30 µL of water. Libraries were matched for concentration and submitted for 50 bp single end sequencing on the Illumina HiSeq 2500.