## **Amino-carboxyl-propyl Reverse Transcription PCR (aRT-PCR): assay for 18S.1248.m1acp3 Ψ**

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Electronic Lab Notebook: <https://www.github.com/ababaian/Crown>

**Assay Notes:**

* It is important to include positive and negative controls in each RNA-extraction and RT-reaction. The exact RT-enzyme and RT-enzyme lot# can cause the baseline ‘misincorporation’ levels to fluctuate. Do not compare samples prepared at different times.
  + Negative Control: Ask your neighbourhood yeast genetics lab if they have a *Tsr3*[KO] strain available and could grow you some for total RNA.
  + Negative Control: Make/grow the HCT116 *TSR3*[KO] cell line as a negative control.
  + Negative Control: In a pinch you can use genomic DNA as a negative control, as rDNA will read out as 100% unmodified ‘rRNA’.
  + Positive Control: HUVEC cells or *any* healthy tissue
* Species compatibility: This assay has been tested to work on Human and Mouse rRNA. The assay will also work on yeast rRNA but the second ‘control’ HinFI cut-site is absent and therefore you can quantify only protected + undigested vs. digested amplicon. In theory this assay will work across all *Eukarya* as long as the HinFI cut-site is conserved, you may need to adjust the primers.

**RNA preparation**

1. Grow cells in preparation for TRIzol extraction.
2. Perform TRIzol extraction by SOP (attached)
   1. Make sure to *INCLUDE* the ‘optional’ 12,000g 5min centrifugation of the lysate which will pellet out ‘fats’, this also removes bulk nuclear DNA and thus contaminate rDNA.
   2. Ensure final RNA pellet is a nice clean white (no pink), or else repeat the RNA wash steps.
   3. Final resuspend RNA in ddH2O
3. Quantify the RNA, input values into spreadsheet template.
4. Starting with 500 ng total RNA, follow the DNAse treatment protocol (TURBO DNAse)
   1. The spreadsheet will automatically calculate the value for each sample, you can change the parameters (in teal) if needed and they will change the calculations.
5. Immediately following DNAse treatment, proceed to RT.

**Reverse Transcription (Superscript III)**

1. From the previous DNase treatment, you’ll load 11 ul of DNAse treated total RNA ( 220 ng).
2. Follow the Superscript III SOP (attached)
   1. Note: using different reverse transcriptases or reaction conditions WILL change the ‘error profile’ of macp and may or may not work. Each reaction condition / enzyme must be tested empirically against a positive and negative control (See Notes).
   2. For First-Strand cDNA synthesis, use only 200 ng of randomer primers and NOT poly-T primers to not bias cDNA for mRNA.
   3. Run incubation at 55’C for 60 minutes

Note: it is good practice to include a “No-RT” control at this point to ensure complete DNase digestion and thus removal of rDNA (which would read out as unmodified rRNA).

Note: I also recommend using the UltraScript 2.0 RT enzyme as it has a better linear range for detecting modification. See *Babaian et al., 2020. Cell Reports.* Figure S2.

**PCR + Restriction Enzyme Digestion**

1. Follow the second-sheet of the attached spreadsheet template for exact reaction conditions.
   1. Use 1 ul cDNA (1:5 dilution in ddH2O) from RT-reaction
   2. PCR Buffer is the standard buffer which comes with Thermo’s Taq polymerase
   3. PCR-grade DMSO is necessary to help melt rRNA
2. Primers with additional RE cut sites. As a PCR control, use any housekeeping gene you like.

>Frag6\_fwd1\_EcoRI

TAGAT GAATTC **GTTCGAAGACGATCAGATACCG**

>Frag8\_rev1\_XbaI

AAGTTG TCTAGA **TCGTTCGTTATCGGAATTAACCAG**

Or

>Frag6\_fwd1

**GTTCGAAGACGATCAGATACCG**

>Frag8\_rev1

**TCGTTCGTTATCGGAATTAACCAG**

1. PCR program
   1. 94’C 5 min
   2. 25x cycles
      1. 94.0’C 30s
      2. 55.0’C 30s
      3. 72.0’C 30s
   3. 72’C 7 min
2. Take 5 ul ( /25 ul PCR reaction) of the amplicon forward for HinFI digestion
   1. Run each sample in triplicate. 2x for the macp-quantification and 1x with no enzyme control.
   2. Using NEB HinFI + CutSmart buffer
   3. Run digestion for 90 minutes
3. Run digested fragments on a 2-2.5% agarose gel in 1x TBE, use the thinnest wells you have to better resolve the lower bands (top-bottom height from viewing angle)
   1. Typically I load 10-15 uL of RE-digested product per lane.
   2. High % agarose gels can be tricky to cast as bubbles readily get stuck in the gel. I prepare the molten agarose in a vacuum-grade Erlenmeyer flask, and vacuum the molten agarose for ~30 seconds when it is hot to degas the solution. Pour carefully while hot and use post-staining with Gel-Red to visualize DNA.
4. Refer to outline attachment
   1. To quantify 1248.macp Modification levels use imageJ
      1. Unmodified or Reference (T) Allele Frequency = ( T band) / (T band + V band)
      2. 100% RAF 🡪 fully unmodified (the first few times you can run genomic DNA in place of cDNA as a control to verify this)
      3. ~45% RAF 🡪my ‘normal’ fully modified signal from HUVEC cells. Although this value WILL vary between RT-batches, therefore it is utmost importance to always run both positive and negative control for EVERY RT-reaction so that you know where you baseline levels are for experimental samples.