CLAIMS

1. A single sample method of identifying ribonucleic acid (RNA) capping and tailing modifications, the method comprising:

(a) providing a sample comprising RNA, optionally having a 5′ cap and/or a 3′ poly A tail, and wherein said sample further comprises a first oligonucleotide probe complementary to a sequence in the 5′ untranslated region (5′ UTR) region of the RNA and a second oligonucleotide probe complementary to a sequence in the 3′ untranslated region (3′ UTR) region of the RNA, wherein the first base of the first oligonucleotide probe binds at the penultimate base of the RNA or adjacent to the penultimate base of the RNA, such as at least 2-10 nucleotides from the penultimate base of the RNA;

(b) annealing the RNA sample with the first oligonucleotide probe and the second oligonucleotide probe;

(c) treating the RNA sample of step (b) with a nuclease to cleave the RNA into cap and tail fragments;

(d) performing liquid chromatography with ultraviolet detection (LC-UV) or LC coupled to mass spectrometry (LC-MS) or LC-UV-MS using the sample of step (c) for:

i. identifying capping species in the cap fragment by measuring retention time of peaks in a chromatogram generated from LC-UV, and/or mass spectra generated by LC-MS or LC-UV-MS;

ii. identifying untailed species in the tail fragment by measuring retention time of peaks in a chromatogram generated from LC-UV, and/or mass spectra generated by LC-MS or LC-UV-MS; and

iii. identifying tailed species in the tail fragment by measuring retention time of peaks in a chromatogram generated from LC-UV, and/or mass spectra generated by LC-MS or LC-UV-MS;

thereby simultaneously identifying RNA capping and tailing modifications in the single sample.

2. A single sample method of simultaneously quantifying RNA capping and tailing efficiency, the method comprising:

(a) providing a sample comprising RNA, optionally having a 5’ cap and/or a 3’ poly A tail;

(b) annealing the RNA sample with a first oligonucleotide probe complementary to a sequence in the 5’ untranslated region (5’ UTR) of the RNA, and a second oligonucleotide probe complementary to a sequence in the 3’ untranslated region (3’ UTR) of the RNA, wherein the first base of the first oligonucleotide probe binds at the penultimate base of the RNA or adjacent to the penultimate base of the RNA, such as at least 2-10 nucleotides from the penultimate base of the RNA;

(c) treating the RNA sample of step (b) with a nuclease to cleave the RNA into cap and tail fragments;

(d) performing liquid chromatography with ultraviolet detection (LC-UV) or LC coupled to mass spectrometry (LC-MS) or LC-UV-MS;

(e) measuring a peak area of each capping species in the cap fragment, and each untailed and tailed species in the tail fragment; and

(f) quantifying a relative amount of each capping species, quantifying a relative amount of untailed species and characterizing poly A tail in the sample of step (d); thereby simultaneously quantifying RNA capping efficiency and tailing efficiency in the sample.

3. The method of claim 2, wherein characterizing the poly A tail comprises (i) mass measurements of poly A tail species, deconvoluting a mass spectrometry signal, thereby providing a mass distribution for tailed species, and (ii) determining based on the corresponding mass distribution, a minimum length, a maximum length, an average length and a degree of polydispersity of the poly A tail.

4. The method of any one of claims 1-3, wherein:

(a) the RNA is *in vitro* transcribed mRNA; and/or

(b) the RNA is obtained from a step of manufacturing, optionally wherein the RNA is obtained from a drug substance (DS) at the final step of manufacturing, or the RNA is obtained from a deformulated drug product (DP).

5. The method of any one of claims 1-4, wherein the RNA is unmodified or modified.

6. The method of any one of claims 1-5, wherein:

(a) the nuclease is RNAse H;

(b) the mass of the cap and the tail fragments is measured by LC-MS;

(c) the capping species, untailed species and tailed species is identified from a UV signal by comparing the retention time of peaks obtained to the retention time of a reference standard;

(d) the capping species is Cap1, Cap0, CapG or uncapped, optionally wherein the uncapped species is GGACA or pppGGACA;

(e) the capping species is m7Gpppm7GGACA, m7GpppGGACA, GpppGGACA, GGACA or pppGGACA, optionally wherein the uncapped species is GGACA or pppGGACA; and/or

(f) the untailed species is UGCAUC, wherein U is unmodified uridine, or the untailed species is U*GCAU*C, wherein U* is N1-methylpseudouridine.

7. The method of any one of claims 1-6, wherein the relative amount of each of the capping species is a percentage of total amount of capping species in the sample calculated by dividing an area under the peak of the capping species of interest over a sum of total areas under the peak representing Cap1, Cap0, CapG and uncapped species, multiplied by 100; and/or the relative amount of untailed species is a percentage calculated by dividing an area under the peak of the untailed species over a sum of areas under the peak of Cap1, Cap0, CapG and uncapped multiplied by 100.

8. The method of any one of claims 1-7, wherein:

(a) the oligonucleotide probe is between about 10 to 40 nucleotides in length;

(b) the oligonucleotide probe comprises RNA and DNA bases;

(c) the oligonucleotide probe comprises RNA and DNA bases in a ratio of about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1 or about 3:1; and/or

(d) the oligonucleotide probe is 10-40 nucleotides long and comprises 4 DNA bases, optionally wherein the oligonucleotide probe complementary to a sequence in the 5' UTR of the RNA is 3'-CCTGTCUAGCGGACCU-5' (SEQ ID NO: 1), wherein italicized CTGT are DNA bases, or optionally wherein the oligonucleotide probe complementary to a sequence in the 3' UTR of the RNA is 3'-GGUCGGAACAGGAUUAUUUUAATTCAA-5' (SEQ ID NO: 2), wherein italicized TTCA are DNA bases.

9. The method of any one of claims 1-8, wherein:

(a) capping and untailed species are quantified in a single LC-UV or LC-MS or LC-UV-MS analysis;

(b) tailed species are characterized in a single LC-MS or LC-UV-MS analysis; and/or

(c) one or more steps is automated.

10. An oligonucleotide that is complementary to a sequence in the 5' untranslated region (5' UTR) of the RNA or 3' untranslated region (3' UTR) of the RNA, wherein the oligonucleotide is between about 10 to 40 nucleotides in length and comprises RNA and DNA bases and wherein the oligonucleotide comprises RNA and DNA bases in a ratio of about 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1 or 3:1.

11. The oligonucleotide of claim 10, wherein:

(a) the oligonucleotide complementary to a sequence in the 5' UTR of the RNA is 3'-CCTGTCUAGCGGACCU-5' (SEQ ID NO: 1), wherein the italicized CTGT are DNA bases; or

(b) the oligonucleotide complementary to a sequence in the 3′ UTR of the RNA is 3′-GGUCGGAACAGGAUUAUUUUAATTCAA-5′ (SEQ ID NO: 2), wherein italicized TTCA are DNA bases.

12. A method of manufacturing RNA having a quantified percentage of capped and untailed mRNA comprising the steps of:

(a) providing *in vitro* synthesized RNA;

(b) annealing the *in vitro* synthesized RNA with a first oligonucleotide complementary to a sequence in the 5′ untranslated region (5′ UTR) of the RNA, and a second oligonucleotide complementary to a sequence in the 3′ untranslated region (3′ UTR) of the RNA, wherein the first base of the first oligonucleotide binds at the penultimate base of the RNA or adjacent to the penultimate base of the RNA, such as at least 2-10 nucleotides from the penultimate base of the RNA;

(c) treating the RNA of (b) with a nuclease to cleave the RNA into cap and tail fragments;

(d) identifying the capping species and untailed species by liquid chromatography with UV detection (LC-UV) and/or identifying and measuring the mass of the capping species, untailed species and characterizing the poly A tail by liquid chromatography coupled to mass spectrometry (LC-MS) and/or liquid chromatography with UV detection and coupled to mass spectrometry (LC-UV-MS); and

(e) quantifying a relative amount of each capping species, quantifying a relative amount of untailed species using LC-UV or LC-MS or LC-UV-MS and characterizing poly A tail using LC-MS or LC-UV-MS simultaneously;

thereby manufacturing RNA comprising a quantified percentage of capped and untailed mRNA.

13. The method of claim 12, wherein:

(a) a 5′ cap is added enzymatically, post transcription to the RNA from step (a), or the RNA does not comprise a 5′ cap;

(b) a 3′ tail is encoded in a plasmid during *in vitro* synthesis or added enzymatically, post transcription to the RNA from step (a), or the RNA does not comprise a 3′ tail; and/or

(c) the RNA is spliced or unspliced.

14. A quality control assay for a manufacturing lot, wherein the assay comprises:

(a) providing a manufactured RNA sample comprising a 5′ cap and/or a 3′ tail;

(b) quantifying RNA capping and tailing efficiency by the method of claim 3 or 4 in the sample;

(c) comparing the capping and tailing efficiency in step (b) to capping and tailing efficiency in a reference sample;

wherein a reduced amount of capping species relative to a reference sample indicates 5′ degradation, and

wherein an increased amount of untailed species and/or a different poly A tail length or polydispersity indicates 3′ degradation,

thereby simultaneously monitoring 5′ degradation and 3′ degradation of an RNA product, and determining product quality of the manufactured lot.

15. The quality control assay of claim 14, wherein the reference sample is a stable RNA sample having greater than 75% integrity, and/or wherein the RNA product is a therapeutic mRNA or an mRNA vaccine.