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Quantification of dsRNA using stable isotope labeling dilution liquid chromatography mass spectrometry

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RATIONALE: Recent developments in RNA interference (RNAi) have created a need for cost-effective and large scale synthesis of double stranded RNA (dsRNA), in conjunction with high throughput analytical techniques to fully characterise and accurately quantify dsRNA prior to downstream RNAi applications.

METHODS: Stable isotope labeled dsRNA was synthesised both *in vivo* (^{15}N) and *in vitro* (^{13}C,^{15}N guanosine-containing dsRNA) prior to purification and quantification. The stable isotope labeled dsRNA standards were subsequently spiked into total RNA extracted from *E. coli* engineered to express dsRNA. RNase mass mapping approaches were subsequently performed using LC-ESI-MS for both the identification and absolute quantification of the dsRNA using the ratios of the light and heavy oligonucleotides pairs.

RESULTS: Absolute quantification was performed based on the resulting light and heavy oligoribonicleotides identified using mass spectrometry. Using this approach we determined that 624.6 ng/µl and 466.5 ng/µl of dsRNA was present in 80 µl total RNA extracted from 10^8 *E. coli* cells expressing 765 bp and 401 bp dsRNA respectively.

CONCLUSIONS: Stable isotopic labelling of dsRNA in conjunction with mass spectrometry enabled the characterisation and quantification of dsRNA in complex total RNA mixtures.

Introduction

Exploitation of the RNAi pathway to block the expression of specific genes holds considerable promise for the development of novel RNAi-based insect management strategies.[1] There are a wide range of future potential applications of RNAi to control agricultural insect pests as well as its use for prevention of diseases in beneficial insects. Recent developments in RNA interference (RNAi) have created a need for cost-effective, large scale synthesis of dsRNA, which in turn requires robust analytical techniques to fully characterise and accurately quantify dsRNA prior to RNAi applications. A wide range of dsRNA products can be generated either via bacterial expression systems, *in planta* or *in vitro* transcription. The development of suitable analytical methods to characterise the dsRNA products remains a significant challenge.

E. coli-mediated delivery of dsRNA has been reported in *C. elegans*, [2-3] planarians, [4] *Entamoeba histolytica* [5] and *Spodoptera exigua* [6]. Furthermore a number of RNAi based insect management strategies have also employed the ingestion of bacteria expressing dsRNA, [7] application of chemically synthesised dsRNA [8] and transgenic plants expressing dsRNA.[6,9] To ensure the RNAi gene silencing using the above approaches it is important to both produce and deliver the required amounts of dsRNA. Therefore the necessary analytical tools to quantify the dsRNA are important to both optimise production strategies and ensure delivery of the required amounts of dsRNA. [10]

Mass spectrometry is a powerful approach for the analysis and direct characterisation of nucleic acids. RNase mass mapping methods have been

performed to identify and characterise a wide range of RNAs.[11-15] Prior to mass spectrometry analysis, purification of the RNA of interest using HPLC is an essential step. For further LC-MS analysis, specific RNase digestions are performed in order to produce smaller oligoribonucleotide fragments, which are then amenable for direct on-line LC separation and MS analysis. RNase mass mapping methods have been widely employed for the identification of RNA and RNA post transcriptional modifications.[11-15] In addition we have recently developed RNase mass mapping approaches to identify and characterise dsRNA.[16]

Recent mass spectrometry studies have focused on the development of more quantitative approaches by using isotopic labelling in conjunction with RNase mapping.[17,22] We were the first to introduce the use of metabolic labelling by utilising *E. coli* to generate both light and heavy labelled RNA prior to LC-MS for the identification and quantification of RNA and RNA modifications.[18] This approach facilitates both the qualitative and quantitative analysis of RNA and RNA modifications. More recent applications have used this method by using a reference material ^{15}N labelled rRNA from *E. coli* to understand the roles that rRNA modifications play inside the living cells.[19] In addition to metabolic labelling, isotope labelling via *in vitro* transcription of RNAs in conjunction with $^{13}C_{10}$ -guanosine triphosphate (GTP), have been used to generate an internal reference to quantitatively characterise rRNA post-transcriptional modifications in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*.[15,20] Furthermore, the ‘‘comparative analysis of RNA digests” (CARD) extends isotopic labelling to tRNA sequencing by labelling the known sequence with $H_2{}^{16}O$, and the unknown sequence with $H_2{}^{18}O$ in order to distinguish an unknown sequence by a mass increase of 2 Da.

[21] More recently, the utilisation of stable isotopic labelled tRNA generated using *in vitro* transcription as an internal standard was developed in order to improve the CARD approach for characterising tRNA was developed (SIL-CARD).[22]

Recent developments in RNA interference (RNAi) have created a need for cost-effective and large scale synthesis of dsRNA, which in turn requires effective analytical techniques to fully characterise and accurately quantify dsRNA prior to RNAi application. Moreover, accurate quantification of dsRNA is important to both optimise production strategies and ensure delivery of the required amounts of dsRNA. UV absorbance spectrophotometry remains one of the most popular methods for the rapid quantification of nucleic acids, however the quantification of individual components in complex mixtures requires their purification prior to analysis. In this study we have utilised stable isotopic labelling using both metabolic labeling and *in vitro* labelling of dsRNA in conjunction with mass spectrometry for the characterisation and absolute quantification of dsRNA in complex total RNA mixtures produced in *E. coli* .

Materials and Methods

Chemicals and reagents

Enpresso® B Defined Nitrogen Free culture medium (BioSilta, UK),^{15}N ammonium sulphate (99%, Cambridge Isotope Laboratories, UK), ^{14}N ammonium sulphate (≥99.0%, Sigma-Aldrich, UK), guanosine-$^{13}C_{10}$, and $^{15}N_5$ 5'-triphosphate sodium salt solution (98 atom % ^{13}C, 98 atom % ^{15}N, 90% CP, Sigma-Aldrich, UK). Ampicillin sodium salt, tetracycline hydrochloride, isopropyl β-D-1-thiogalactopyranoside (IPTG), triethylammonium acetate (TEAA), 1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP) were all purchased from Sigma-Aldrich, UK.

HPLC grade water, methanol, and acetonitrile were obtained from Thermo Fisher Scientific, UK. RNase A was from Ambion, UK. Synthetic genes were synthesised via GeneArt (Invitrogen Life Technologies, UK) and the designed primers were purchased from MWG Eurofins, UK. Purelink Genomic DNA Mini Kit and PCR master mix were obtained from Thermo Fisher Scientific, UK.

In vitro transcription of dsRNA

PCR amplified DNA was used as the template for *in vitro* transcription reactions in conjunction with HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, UK). For isotope labelling 2.0 µL of each the NTPs (10 mM) were used where GTP was replaced with guanosine-$^{13}C_{10}$, $^{15}N_5$ 5'-triphosphate (Sigma-Aldrich, UK). 2 µL of 10X reaction buffer, 1µg DNA template and 2 µL HiScribe T7 polymerase were added to 20 µL RNase-free water and incubated at 37 °C for 4 hours.

Expression of dsRNA in *E. coli* HT115 (DE3)

E. coli HT115 (DE3) cells (Cold Spring Harbor Laboratory, NY, USA) were used for the transformation of plasmids carrying an insert to generate dsRNA products of 765 bp and 401 bp respectively. Pre-cultures were prepared by inoculating a single colony from the transformed cells into 5 mL of LB medium containing tetracycline (10 µg/mL) and ampicillin (100 µg/mL) or tetracycline (10 µg/mL) and kanamycin (50 µg/mL), before incubating with vigorous shaking at 37°C overnight until the OD_{600} reached 0.6. For metabolic isotope labelling a defined nitrogen-free medium was prepared starting with 45 mL of sterile water with two tablets of Enpresso B Defined Nitrogen Free medium, adding 3 mL of $(^{14}NH_4)_2SO_4$ or $^{15}NH_4Cl$ (40 g/L, final concentration 2.5 g/L) to the defined media which contained tetracycline (10 µg/mL) and ampicillin (100 µg/mL) or tetracycline (10 µg/mL) and kanamycin (50 µg/mL). 5-10% of overnight inoculum were used in 50 mL cultures in both light and heavy media. The cultures were incubated with shaking at 37°C until an OD_{600} of 0.6 was reached. IPTG was added to a final concentration of 1 mM and the cultures incubated for a further 2 hours.

RNA extraction and purification of dsRNA

RNA extractions were performed using RNASwift as previously described.[7] Purification of ^{15}N dsRNA standard from total RNA was performed by adding 0.1 µg of RNase A followed by 10 min incubation at 37°C. Prior to solid phase extraction, 300 µl of IPD buffer (containing 33% isopropanol and 33% DMSO) was added and the mixture loaded into the column followed by centrifugation at 13,000 rpm for 1 min. The flow-through was discarded and 700 µL of wash buffer (10 mM Tris-HCl pH 7.5

+ 80% EtOH) was added and centrifuged for 1 min. The dsRNA was eluted by adding 80 µL nuclease free water. Quantification using a Nanodrop 2000 UV visible spectrophotometer (Thermo Fisher Scientific) using an extinction coefficient of 0.021 $(\mu g/mL)^{-1}$ cm^{-1} which corresponds to 1 A_{260}= 46.52 µg/ml. For RNase digestion, 0.1 µg RNase A was added to 1 µg of RNA sample. Digestion was performed for an hour at 37°C.

Ion pair reverse phase chromatography

IP RP HPLC (Agilent 1100 series HPLC, Germany) and a ProSwift RP-1S column (Thermo Fisher Scientific, UK) were used to analyse all samples of purified intact dsRNA. Chromatograms were acquired at 260 nm. Binary eluent mode was applied (buffer A: 0.1 M triethylammonium acetate (TEAA) pH 7.0, 0.01% acetonitrile; and buffer B: 0.1 M TEAA, 25% of acetonitrile) at flow rate of 1 mL/min and a column temperature of 50°C. The analyses were performed using a linear gradient as follows: 22% buffer B held for 2 min, followed by an increase to 25% buffer B, followed by a linear extension to 62% buffer B over 15 min, and finally a linear extension to 73% buffer B in 2.5 min.

Liquid chromatography electrospray ionisation mass spectrometry

The RNase digestion products were analysed on a maXis ultra high-resolution time-of-flight (UHR-TOF) mass spectrometer (Bruker Daltonics, Germany) interfaced with a liquid chromatography system (U3000, Thermo Scientific, UK). HPLC was performed using an Accucore C18 column (150 mm × 2.1 mm ID, Thermo Fisher), 0.1 ml/min at 30 °C. Buffer A, 20 mM TEAA, 80 mM 1,1,1,3,3,3,-hexafluoro-2-

propanol (HFIP), and buffer B 20 mM TEAA, 80mM HFIP, and 50% ACN. The gradient conditions used were: 10% buffer B held for 2 min, followed by a linear increase to 20% B in 20 min, followed by a linear extension to 25% B over 10 min, and finally a linear extension to 80% B in 2 min. All analyses were performed in negative ion mode at a mass range of 300-2500 m/z. The ion source voltage was set to -2000V. The capillary temperature was maintained at 300°C with a N_2 nebuliser gas pressure of 0.4 bar at a flow rate of 6.0 L/h.

A list of theoretical monoisotopic masses of RNA oligoribonucleotides fragments (obtained from RNase A digestion) was compiled from calculations using Mongo Oligo Mass Calculator (http://library.med.utah.edu/masspec/mongo.htm). All possible chemical terminals were selected during data processing, including 5'-OH, 5'-phosphate, 5'-cyclic phosphate and 3'-OH 3'-phosphate, 3'-cyclic phosphate. The resulting theoretical monoisotopic masses were utilised to calculate the heavy isotope oligoribonucleotide monoisotopic mass using the elemental or base composition of the oligoribonucleotide sequences in conjunction with the calculated number of nitrogens or guanosines. IsoPro 3.1 software was used to calculate theoretical relative intensities of the oligoribonucleotide isotopomers. For the quantification of oligoribonucleotide fragments using stable isotope labelling, samples consisting of differing amounts of heavy and light isotopes were mixed by spiking known concentrations of purified ^{15}N dsRNA into unknown concentrations of ^{14}N labelled total RNA. Prior to the experiment, all the samples were quantified using UV spectrophotometry followed by LC–MS analysis. An extracted ion chromatogram (XIC) was constructed for each oligoribonucleotide. This tool was used to check for chromatographic shifts between heavy and light versions of the same

oligoribonucleotide. Absolute quantification was performed by measuring the ratio and peak areas of both light and heavy samples using DataAnalysis software (Bruker Daltonics) across 10 different oligoribonucleotides. This was performed for 3 different experimental samples, each of which had different amounts of ^{15}N internal dsRNA standard present, enabling a final absolute concentration of dsRNA represented as an average across the 3 different samples.

Results and discussion

Synthesis and purification of isotope labelled dsRNA

Most RNAi research in insects has been performed using dsRNA constructs of between 100–800 bp [10,23] and a minimum length of approximately 60 bp for effective RNAi in several insects has been demonstrated.[21,24,25] The use of larger dsRNA molecules generates many siRNAs via dicer cleavage, which contributes to the RNAi response and prevents the resistance due to the polymorphism variation encoded by nucleotide sequences. Therefore we chose to generate dsRNAs corresponding to this size range.

To characterise and quantify the dsRNA expressed in *E. coli,* two different strategies were employed. ^{15}N dsRNA was generated *in vivo* by growing *E. coli* HT115 (DE3) cells (transformed with plasmids to express dsRNA) on heavy (^{15}N) media. In addition, stable isotope labelled RNAs were also synthesised *in vitro* using *in vitro* transcription in conjunction with $^{13}C_{10,}{}^{15}N_5$ (GTP) to generate $^{13}C_{10,}{}^{15}N_5$ guanosine-containing RNA as previously.[6,14] Following *E. coli* growth on the ^{15}N media, dsRNA was extracted , treated with RNAase A to remove the background rRNA/tRNA and purified using solid phase extraction[7] prior to analysis using IP RP HPLC (see Figure 1A). The results show the successful synthesis and purification of the dsRNA (765 bp) from *E. coli*; no significant contaminating rRNA was present. Following *in vitro* synthesis of the isotope labelled dsRNA (401 bp), purification was performed using solid phase extraction to remove excess NTPs prior to analysis using IP RP HPLC (see Figure 1B). The IP RP HPLC shows that no significant contaminating ssRNA or NTPs were present. Quantification of the purified isotope labelled dsRNA standards was subsequently performed using UV spectrophotometry by a Nanodrop

spectrophotometer. Accurate quantification of the internal isotope labelled dsRNA standards is important, as this value directly determines the quantification of the dsRNA in the biological samples. UV spectrophotometry was used to accurately determine the concentration of the dsRNA standards, therefore it is important to ensure the dsRNA is purified and accurate extinction coefficients are used for dsRNA. We have previously measured the hypochromicity of dsRNA to accurately determine the overall extinction coefficient and mass concentration/A_{260} (46.52 µg/ml/A_{260}).[26] This value was subsequently used for the quantification of dsRNA using UV spectrophotometry.

Characterisation and quantification of dsRNA using stable isotopic labelling in conjunction with RNase mass mapping

To characterise and quantify dsRNA expressed in *E. coli*, total RNA was extracted from ^{14}N *E. coli* HT115 cells expressing a 765 bp dsRNA and 401 bp dsRNA and analysed using IP RP HPLC (see Figure 2). The results show the expected chromatogram, highlighting the presence of the abundant tRNA/rRNA together with dsRNA. Direct analysis using UV spectrophotometry cannot accurately determine the amount of dsRNA present in these complex mixtures. Therefore, following validation of the expression and extraction of the dsRNA in complex RNA mixtures extracted from *E. coli*, the stable isotope labelled dsRNA standards previously generated were spiked into these samples prior to LC MS analysis.

A range of amounts of the *E. coli* ^{15}N purified dsRNA (765 bp) were combined with ^{14}N total RNA extract containing the 765 bp dsRNA in conjunction with tRNA/rRNA

and subsequently digested using RNase A. The oligoribonucleotide fragments were analysed using LC-ESI-MS. The application of stable isotope labelling enables the identification of oligoribonucleotides generated from the dsRNA in complex mixtures. All corresponding oligoribonucleotides from the dsRNA appear as light and heavy pairs in contrast to the abundant oligoribonucleotides generated from the background rRNA and tRNA present in the total RNA extract. An example of the MS spectra obtained from an oligoribonucleotide generated from rRNA is shown in Supplementary Figure 1, the absence of the associated heavy oligoribonucleotide enables identification of oligoribonucleotides generated from rRNA not dsRNA. Therefore, this approach simplifies the identification and quantification of the dsRNA in complex RNA mixtures as light and heavy pairs that can readily be identified in complex MS chromatograms. Figure 3A shows the identification of ^{14}N and ^{15}N sense strand oligoribonucleotides (from the dsRNA), AAGAUp and GAAGGUp detected in varying amounts of spiked ^{15}N dsRNA standard. Absolute quantification was then performed by measuring the ratio and peak areas of both light and heavy pairs for 10 different identified oligoribonucleotides across 3 different amounts of dsRNA standard. The results are summarised in Figure 3B/C/ Supplementary Table I. Absolute quantification of ^{14}N dsRNA in the total RNA resulted in 466.5 ng ± 18.7 ng/µL of dsRNA in 80 µl total RNA extracted from 10^8 *E. coli* cells dsRNA using an average across the 3 different experiments.

Following quantitative analysis of dsRNA using the metabolic isotopic labelling approach in conjunction with LC-MS, we further demonstrated the use of *in vitro* labelled dsRNA (^{13}C,^{15}N guanosine dsRNA) as the internal standard to characterise and quantify dsRNA expressed in *E. coli*. Total RNA was extracted from *E. coli*

HT115 cells expressing a 401 bp dsRNA and analysed using IP RP HPLC (see Figure 2B). A range of amounts of $^{13}C,^{15}N$ guanosine dsRNA (401 bp) were added prior to RNase A enzymatic digestion and analysis of the oligoribonucleotide using LC-ESI-MS as previously described. Figure 4A shows the identification of light and heavy oligoribonucleotides. The representative mass spectrum of the doubly charged unique sense and antisense strands oligoribonucleotide, AGAAGAUp and GGAAGGUp detected in varying amounts of spiked heavy dsRNA standard. Absolute quantification was then performed by measuring the ratio and peak areas of both light and heavy pairs for 10 different identified oligoribonucleotides across 3 different amounts of standard. The results are summarised in Figure 4B/C/ Supplementary Table II. Absolute quantification of the ^{14}N dsRNA present in the total RNA revealed 624.6 ±14.24 ng/µL of dsRNA in 80 µl total RNA extracted from 10^8 *E. coli* cells expressing dsRNA using an average across the 3 different experiments.

Conclusions

Stable isotopic labelling of dsRNA both *in vitro* and *in vivo* was used in conjunction with mass spectrometry for the characterisation and quantification of dsRNA in complex total RNA mixtures. This approach enables the accurate quantification of dsRNA from a complex mixture without the need to purify the dsRNA from contaminating rRNA and NTPs that prevent accurate analysis using UV spectrophotometry. Furthermore, the presence of abundant tRNA and rRNAs present in the HPLC chromatogram can limit the accurate quantification of the dsRNA directly from the HPLC chromatogram in situations where the dsRNA co-elutes with the rRNA, or multiple heterogeneous dsRNA are synthesised.

Stable isotope labeled dsRNA standards were synthesised (^{15}N and ^{13}C,^{15}N guanosine containing) *in vivo* and *in vitro* prior to purification and quantification. The stable isotope dsRNA standards were subsequently mixed into RNA extracted from *E. coli* that was engineered to express dsRNA prior to RNase digestion and LC-ESI-MS analysis. Absolute quantification was performed based on the resulting light and heavy oligoribonicleotides identified using mass spectrometry. Using this approach we determined that 624.6 ng/µL and 466.5 ng/µL of dsRNA was present in 80 µL total RNA extracted from 10^8 *E. coli* cells expressing 765 bp and 401 bp dsRNA respectively.

352 **Legends to Figures:**

353 Figure 1. IP RP HPLC analysis of purified heavy stable isotope labelled dsRNA. (A)
354 Purified ^{15}N dsRNA from *E. coli* HT115 (DE3) cells expressing a 756 bp dsRNA, 1.9
355 µg of dsRNA was injected. (B) Purified ^{15}N dsRNA (401 bp) ^{13}C,^{15}N guanosine
356 containing dsRNA synthesised using *in vitro t*ranscription. 2.0 µg was injected.

357

358 Figure 2. IP RP HPLC analysis of total RNA extracted from *E. coli* HT115 (DE3) cells
359 expressing dsRNA. (A) Total RNA extracted from *E. coli* HT115 (DE3) cells
360 expressing a 756 bp dsRNA. The rRNA, tRNA and dsRNA are highlighted.
361 Approximately 7 µg of total RNA was injected and analysed. (B) Total RNA extracted
362 from *E. coli* HT115 (DE3) cells expressing a 401 bp dsRNA. The rRNA, tRNA and
363 dsRNA are highlighted. Approximately 11 µg of total RNA was injected.

364

365 Figure 3. Absolute quantification of dsRNA using a stable isotope labelled dsRNA
366 standard generated *in vivo* in conjunction with mass spectrometry. (A) MS spectra of
367 the oligoribonucleotide AAGAUp (sense strand), GAAGGUp (antisense strand)
368 across varying light:heavy ratios. (B) Light to heavy ratios of ten different
369 oligoribonucleotides across varying amounts of isotope labelled dsRNA standard. (C)
370 Average light:heavy ratios with the error bars representing standard deviation.

371

372 Figure 4. Absolute quantification of dsRNA using *in vitro* transcribed stable isotope
373 labelled dsRNA. (A) MS spectra of *the* oligoribonucleotides, AGAAGAUp and
374 GGAAGGUp oligoribonucleotides across varying light:heavy ratios. (B) Light to
375 heavy ratios of ten different oligoribonucleotides across varying amounts of isotope

labelled dsRNA standard. (C) Average light:heavy ratios with the error bars representing standard deviation.

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