ABSTRACT

Methylation of RNA by methyltransferases is a phylogenetically ubiquitous post-transcriptional modification that occurs most extensively in transfer RNA (tRNA) and ribosomal RNA (rRNA). Biochemical characterization of RNA methyltransferase enzymes and their methylated product RNA or RNA–protein complexes is usually done by measuring the incorporation of radiolabeled methyl groups into the product over time. This has traditionally required the separation of radiolabeled product from radiolabeled methyl donor through a filter binding assay. We have adapted and optimized a scintillation proximity assay (SPA) to replace the more costly, wasteful and cumbersome filter binding assay and demonstrate its utility in studies of three distinct methyltransferases, RmtA, KsgA and ErmC'. In vitro characterization of RMT usually requires the separation of radiolabeled product from radiolabeled methyl donor through a filter binding assay. We show that this method is suitable for quantitating extent of RNA methylation or active RNA methyltransferase, and for testing RNA-methyltransferase inhibitors. This assay can be carried out with techniques routinely used in a typical biochemistry laboratory or could be easily adapted for a high throughput screening format.

INTRODUCTION

Post-transcriptional methylation of RNA by RNA methyltransferases (RMT) is universal and occurs to its greatest extent in transfer RNA (tRNA) and ribosomal RNA (rRNA). All of these methylations require the S-adenosyl-methionine (SAM) cofactor as methyl donor. Measurements of labeled product may be used to kinetically and enzymologically characterize the enzyme and determine the kinetics and potential inhibitors or to assess the extent and consequences of product RNA methylation.

We have been studying three different classes of rRNA methyltransferase: one is the housekeeping protein, KsgA (1); the other two, RmtA (and related RMT family members) (2) and ErmC' (3), confer aminoglycoside and macrolide antibiotic resistance, respectively, on their bacterial hosts. KsgA converts both A1518 and A1519 (Escherichia coli numbering) of 16S rRNA in the 30S ribosomal particle to N6,N6-dimethyladenosines (4) by catalyzing the transfer of four methyl groups from SAM to each 16S RNA molecule. RmtA and related members of its enzyme class catalyze transfer of one methyl group to the N7 position of G1405 (E. coli numbering) of 16S rRNA in the 30S ribosomal particle (5). ErmC' catalyzes the mono- or di-methylation of A2058 (E. coli numbering) in the 23S rRNA of the 50S ribosomal particle (6).

In vitro characterization of RMT usually requires the use of 3H-methyl-SAM (radiolabeled on the methyl group) as cofactor reactant with the RNA substrate, and a method of separating 3H-methyl-RNA product from unreacted 3H-SAM. The separation is usually accomplished by filter binding (7) or with a spin column (8). While these methods are widely used, they suffer from a variety of shortcomings, including empirical determination of wash conditions, variation in deposition efficiencies, large volumes of radioactive waste and the inability to scale up to a large number of parallel measurements used in HTS. One way to circumvent these deficiencies has been to use fluorescence-based assays that measure the S-(5'-adenosyl)-l-homocysteine (SAH) product rather than the methylated RNA. This type of assay has the advantages of being adaptable for high-throughput screening (HTS) and not requiring radiolabeled reagents, but it does not directly measure the methylated product, which is often the objective of this type of assay. One version of this assay used SAH antibodies with conjugate tracers to measure activity, but high cross-reactivity of the anti-SAII antibody with SAM (9) limited its use to...
low concentrations of SAM (3 μM). Other indirect assays using coupled enzymes to convert SAH to detectable products (10–12) are potentially ambiguous, since test compounds (e.g. inhibitor screens) could inhibit these enzymes but not the methyltransferase.

We have developed a scintillation proximity assay (SPA) for the assay of RMT using commercially available yttrium silicate scintillant (YSi) beads that capture DNA and RNA by non-specific electrostatic binding. In our application, radiolabeled, methylated RNA product bound to the bead activates the scintillant whose emission can be measured and quantitated. The intrinsic physical separation of the radiolabeled, methylated RNA product from radiolabeled SAM reactant permits the accurate direct quantitation of methylated RNA product without the need for a separation step. This method should be generally applicable to assay of RNA-methyltransferases and their products and adaptable to HTS.

**MATERIALS AND METHODS**

**Protein expression and purification**

KsgA was expressed and purified as described (7). RmtA and ErmC’ were expressed from cloned, synthetic genes inserted into pET15b plasmids, rmtA in Rosetta 2 (DE3) cells (Novagen) and ermC in BL21 (codon+ DE3-RIL) cells (Stratagene). All three enzymes were purified on a HiTrap Ni-chelating column (Amersham) to >95% homogeneity on SDS gel.

**30S purification**

Wild type and ksgR strains of *E. coli* were provided by Dr Heather O’Farrell and 30S subunits were prepared according to a previously described method (7). Concentration of 30S was determined by multiplying OD260 by 6 pmol/ml.

**30S particle methylation reaction**

A standard 50 μl reaction contained 10 pmol enzyme (RmtA or KsgA), 10 pmol 30S (wild type for RmtA, unmethylated ksgR for KsgA), 0.02 mM 3H-methyl-SAM (780 cpm/pmol) (Perkin Elmer) and reaction buffer incubated at 37°C for a specified time. Prior to mixing the reactants, 30S particles were heated at 42°C for 5 min to anneal subunits into a homogenous conformation. Reactants were mixed and the reaction initiated by addition of SAM. Background reactions were performed in the same way without enzyme. In reactions with RmtA and for its background measurement, reaction buffer (buffer R) consisted of 40 mM Tris, pH 7.2, 40 mM NH4Cl, 8 mM MgOAc and 1 mM DTT. Reaction buffer for KsgA reactions and for its background measurement was 40 mM Tris at pH 7.4, 40 mM NH4Cl, 4 mM MgOAc, 6 mM 2-mercaptoethanol.

At the end of the reaction time, the 50 μl reaction volume was removed and added to clear 1.5 ml vials containing 180 μl deionized water, 10 μl (100 mg/ml) YSi binding beads (cat. RPQ0013, GE Life Sciences) and 10 μl 100 mM unlabeled SAM (Sigma) and mixed thoroughly by pipette. Vials were then incubated in the dark for 40 min with mixing by inversion at 20 and 40 min and then centrifuged at 13 000 rpm for 3 min, placed in the mouth of a 15 ml scintillation vial, and counted in a Packard 1500 Tri-Carb Liquid Scintillation Analyzer. For experiments to optimize bead concentration (Figure 1A) and water dilution (Figure 1B), sample/bead mixtures were pipetted into the bottom of the scintillation vial and allowed to settle for an additional 20 min in the dark without mixing before being counted. Time-course assays of samples before and after centrifugation (Figure 1C) were done in one vial in volumes sufficient for eight samples. Samples of 50 μl were taken at each of the eight designated time points and measured by the standard SPA method with the additional step of counting samples just prior to centrifugation. Signal and background reactions were measured in triplicate at 1, 2, 4, 8, 16, 32, 64 and 128 min.

Parallel time course assays (Figure 2) were done as above except the reaction volume was sufficient for 16 samples for signal reactions and 6 samples for background reactions. Samples of 100 μl were removed at each time point, quenched with 20 μl 100 mM unlabeled SAM, and divided into two 60 μl aliquots for determination by the SPA and filter binding methods. Signal reactions were measured at 1, 2, 4, 8, 16, 32, 64 and 128 min, while background reactions were measured at 1, 8 and 128 min, all in triplicate.

Excel was used to obtain a linear fit of data in Figures 3, 6 and background data in Figure 2, and a logarithmic fit of signal data in Figures 2 and 4.

Inhibition studies were done using RmtA and wild-type 30S in a triple volume reaction. Inhibitor or solvent was added at a specified concentration prior to the addition of SAM to initiate the reaction. Reactions were stopped after 8 min and dispensed in equal volumes into three vials with YSi beads. Sinefungin (Sigma) was dissolved in deionized H2O, SAH (Sigma) was dissolved in DMSO (American Bioanalytical). Data in Figure 7A and B were fitted with a sigmoidal dose–response curve provided in Sigma Plot 8.0; IC50 values were calculated as described (13).

**Extraction of 16S and 23S rRNA from 30S and 50S particles**

Methylated 16S rRNA after reaction with KsgA or RmtA was extracted with buffer saturated phenol (Invitrogen) and precipitated with ethanol before dilution and counting. 23S rRNA was similarly extracted from 50S particles with phenol prior to methylation by incubation with ErmC’.

**RESULTS AND DISCUSSION**

**Optimization of SPA assay conditions**

*Bead concentration affects the signal to noise ratio.* To optimize the YSi bead concentration, we tested signal and background reactions with different amounts of YSi beads (Figure 1A). We found that for our assay conditions, 1 mg of YSi beads gave the highest signal:noise ratio with a signal greater than 6000 cpm. These experiments also suggest that the apparent failure to reach saturation of the beads with bound, methylated 30S
riboosomal particles is due to the non-proximity effect (NPE) as bead concentration increases (Figure 1A). The NPE arises from excitation of beads by radiolabeled substrate that is within the path length of the isotope’s β-particle (1.5 μm for 3H; GE Health Sciences), but is either free in solution or bound to other nearby beads. Bead concentrations should be optimized for specific applications.

Minimizing background from 3H-methyl-SAM. 3H-methyl-SAM alone in reaction buffer produced surprisingly high counts (Figure 1B) that would raise background levels in the assay. Addition of cold 30S (10 pmol) ribosomal particles to the incubation of 3H-methyl-SAM with YSi beads lowered the counts, particularly for vials with smaller amounts of YSi beads, indicating that SAM and 30S ribosomal particles compete for sites on the YSi beads.

A significant diminution of background counts from 3H-methyl-SAM binding to the beads was achieved
because the higher affinity 30S ribosomal particles in the
3
This indicated that the major contributor of 3
RNA. Formulas derived from Figure 3 were used to convert SPA
Figure 4.
Extent of methylation. Time courses measured by SPA in this
curve are expressed in number of methyl groups incorporated into
by dilution of the sample with 3 ml of water (Figure 1B).
This indicated that the major contributor of 3H-methyl-
SAM to the background counts is through the NPE and
not through binding to the beads. Dilution of the reaction
solution with water to a volume of ~250 µl (the well
volumes of a typical 96-well plate) on addition to the YSi
beads almost entirely eliminated this background.
Attempts to lower background counts by dilution with
solutes (adenosine, unlabeled SAM, spermine, HCl or
MeOH) showed that only unlabeled SAM lowered
background without lowering signal. We conclude that
MeOH) showed that only unlabeled SAM lowered
background counts did not increase
5:1). Counts from signal reactions more than
above background than the filter binding method, signals from
filter binding technique. Both sets of experiments
showed the expected exponential curve with an early
linear phase extending up to about 5 min and an extended
asymptotic plateau. Although the SPA produced a higher
background indicate that the high background does not
compromise the results. The SPA background increased
slightly over time in a linear fashion. This could be due to
degradation of 3H-methyl-SAM to a radiolabeled product
with higher affinity for SPA beads.

Sample centrifugation increases the signal to noise ratio. We found that centrifuging samples just prior to
scintillation counting improved the signal:noise significantly (>5:1). Counts from signal reactions more than
doubled, while those of background reactions increased
only slightly (Figure 1C). This phenomenon may also be
due to the NPE, arising in this case from more tightly
packed radiolabeled 30S particle-bound beads exciting
proximal YSi beads within the 1.5 µm path length of the
tritium β-particles. Background counts did not increase
significantly on centrifugation, consistent with the low
level of bound radiolabeled substrate. More importantly,
centrifugation of the beads after product binding also
reduced variability among replicate samples, possibly by
detaching beads bound to the walls of the vial and packing
them more uniformly. A centrifugation step can be incor-
porated into HTS experiments by centrifuging 96-well
plates in a special rotor as described in a similar SPA
HTS assay (14).

Binding of methylated 30S ribosomal particles to YSi
beads is instantaneous. Incubation time for YSi beads
with product mix was optimized to ensure maximum
capture of 30S ribosomal particles. A large batch
(70 pmol) of 30S rRNA particles was methylated by
RmtA for 1 h and separated into individual 10 pmol por-
tions to test the effect of different incubation times (0, 20,
40, 60, 80, 100 and 120 min). YSi beads (density = 4.1 g/
cm3) quickly settle in solution, so beads were thoroughly
mixed with a pipette on addition of the reaction sample
time 0) and then mixed by inversion every 20 min for the
specified time. The signal from time 0 was approximately
the same as the other incubation times (data not shown),
indicating that 30S binding occurs almost instantaneously
and is complete with the first mixing. For convenience, all
succeeding samples were incubated for 40 min; i.e. mixed
by pipette initially and again at 20 and 40 min before
centrifugation.

With these optimized conditions, we attained minimum
count levels of ~3000 cpm and signal:noise ratio of ~5:1,
which are the recommended benchmarks for this bead-
based assay (GE Health Sciences). While these conditions
are useful as a guide to implementing the assay, optimization
should be done for applications to different assay
systems.

SPA vs. filter-binding method in measurement of
time courses and extent of methylation
Our standard filter-binding assay for measurement of
methyltransferase activity is based on an earlier method
(15) in which reaction solutions are pipetted onto cationic
filter discs, washed twice with ~200 ml of 5% TCA and
rinsed with ~3 ml ethanol. After drying for an hour, filter
discs are placed in 3 ml of scintillation fluid for counting.

We compared this method with the SPA method in par-
allel time-course experiments. Using our standard opti-
mized reaction conditions, we ran time courses of reaction for RmtA and KsgA, including separate back-
ground measurements (Figure 2). For each time point,
double the normal amount was removed from the reac-
tion, quenched and divided so that equal amounts were
measured by either the SPA method or the traditional
filter binding technique. Both sets of experiments
showed the expected exponential curve with an early
linear phase extending up to about 5 min and an extended
asymptotic plateau. Although the SPA produced a higher
background than the filter binding method, signals from
the SPA were greater than for filter binding after back-
grounds were subtracted. The consistent values of SPA
background indicate that the high background does not
compromise the results. The SPA background increased
slightly over time in a linear fashion. This could be due to
degradation of 3H-methyl SAM to a radiolabeled product
with higher affinity for SPA beads.

The data from these parallel time courses allowed us to
calculate the amount of 3H-methyl transferred to RNA
from the SPA measurements. In the filter binding
method, the extent of methylation can be easily deter-
mined from knowledge of the amount of RNA substrate
in the assay and the measurable specific activity of the
3H-methyl-SAM used. However, in the SPA format, the
amount of 3H-methyl-SAM used cannot be directly mea-
sured with a specific-activity test, because the different

Figure 4. Extent of methylation. Time courses measured by SPA in this
figure are expressed in number of methyl groups incorporated into
RNA. Formulas derived from Figure 3 were used to convert SPA
measurements into ‘filter binding equivalent’ values for quantitation
of methyl group incorporation. RmtA + wild-type 30S (maroon); KsgA + κsg6 30S (orange).
binding affinities of $^3H$-methyl SAM and $^3H$-methyl RNA to the SPA beads will yield a different response for the same amount of $^3H$-methyl group.

This problem is circumvented by plotting the SPA and filter binding responses from the same samples and obtaining the linear equation that relates them (Figure 3). SPA measurements can then be converted into 'filter binding equivalent' values, from which RNA methylation is quantified (Figure 4) using the specific activity of $^3H$-methyl-SAM. The number of methyl groups incorporated into RNA by KsgA or RmtA determined from the SPA assay is consistent with full methylation of the 10 pmol of 30S substrate by the two enzymes: 10 pmol of methyl group for RmtA and 40 pmol for KsgA.

Performing a parallel filter binding assay may be needed only when quantifying methyl group incorporation. In this case, once a correlation of SPA and filter binding measurements is established for a particular enzyme reaction, subsequent SPA measurements could be quantified by use of the previously obtained SPA to filter binding conversion factor and the measured specific activity of the $^3H$-methyl-SAM.

**SPA analysis of extracted $^3H$-methyl RNA**

The experiments described above measure methylation of 30S subunit particles that bind intact to YSi beads. We also tested the SPA assay format on methylated RNA extracted with phenol to remove proteins. Phenol-extracted, methylated 16S rRNA from RmtA or KsgA catalyzed reactions was tested for binding to YSi beads in the SPA assay. Signals for both reaction products increased linearly with amount of extracted $^3H$-methyl-RNA up to 10 pmol (Figure 5). The counts measured for 10 pmol of extracted RNA were higher than those for the same amount of 30S particles, suggesting that free RNA has a higher affinity for YSi beads than RNA bound to protein in the 30S subunit or that the NPE is larger for bound RNA in the absence of protein. The background for the extracted RNA measurements was essentially nil, since all $^3H$-methyl SAM is washed away in the extraction process.

**SPA assay for methylation of free RNA substrate**

To verify that this SPA procedure can measure methylation of an RNA substrate alone, parallel time course experiments were performed as above using the ErmC' enzyme and phenol-extracted 23S rRNA from 50S ribosomal particles. The kinetics of this reaction differ from those for KsgA and RmtA, but with 10 pmol of E. coli 23S rRNA substrate, we measure ErmC'-catalyzed incorporation of 8 pmols of methyl group (data not shown), in close agreement with the results of Denoya and Dubnau (16) for this reaction. Furthermore, the correlation of SPA and filter-binding values for the ErmC'-catalyzed reaction is closely similar to that obtained for the KsgA and RmtA assays.

Use of a similar YSi SPA bead to monitor methylation of tRNA has been reported (17) for considerably different assay conditions, suggesting that this method can be applied widely to both methylated RNA and RNA–protein complexes.

**Figure 5.** Free $^3H$-methyl-RNA measured with YSi SPA beads. RNA extracted from 30S subunits following a 2-h reaction with RmtA + wild-type 30S (maroon) or KsgA + $ksg^R$ 30S (orange), and incubated with different amounts of YSi beads. RNA concentration was determined by measuring the OD$_{260}$ following extraction. RNA (10 pmol) extracted from reactions without enzyme were used as a negative control. Data are for single samples.

**Figure 6.** Dependence on enzyme concentration. 30S ribosomal particles (10 pmol) were incubated with RmtA (maroon) for 8 min and 10 pmol $ksg^R$ 30S ribosomal particles were incubated with KsgA (orange) for 3.5 min. Samples were done in triplicate and the average plotted with error bars of ± 1 SD.

**Dependence on enzyme concentration**

Based on the time course, we took early samples (8 min for RmtA, 3.5 min for KsgA) of reactions carried out with varying amounts of RmtA or KsgA (Figure 6) to determine the dependence on enzyme concentration. Under the reaction conditions used, the activity measured from the SPA assay is linear for enzyme concentrations up to at least 40 nM (10 pmol enzyme in the reaction), validating the use of the assay for determining enzyme concentrations.

**Inhibitor assays**

We tested the SPA assay for RmtA inhibition by the known methyltransferase inhibitors sinefungin and SAH (Figure 7A). Both compounds inhibited RmtA in a concentration-dependent manner with calculated IC$_{50}$ values of 19.95 ± 0.09 μM for sinefungin and 9.33 ± 0.07 μM for SAH. We tested the effect of DMSO in the inhibition reactions, since this solvent is often used in HTS assays to dissolve test compounds (Figure 7B).
RmtA in the presence of DMSO appeared to be fully active up to 15% (V/V), but the response from SPA beads dropped off at ≥20% DMSO. Whether the drop in signal was from the effect of DMSO on enzyme activity or on the binding of methylated RNA product to, or response of, the SPA beads was not determined.

CONCLUSIONS

Yttrium silicate nucleic-acid-binding SPA beads are shown here to be a useful method for measuring methylation of rRNA and almost certainly for other RNA and RNA–protein complexes. This assay method gives signal:noise ratios > 5:1 under our optimized conditions and can be used to measure methylation of RNA alone or in 30S ribosomal particle complex with protein. While optimization of conditions for a specific assay and its products is advisable, all assays of RNA methylation will involve the common step of non-specific binding of the RNA product to the YSi beads. Therefore, the conditions described here are likely to be a good starting point for any further optimization. Through the use of a standard reference curve relating SPA counts to counts from the traditional filter binding assay, the SPA assay can give reproducible and accurate measures of the stoichiometry of methyl group incorporation. While the creation of this standard reference curve adds a step to the assay when methylation stoichiometry is to be determined, it is not necessary for routine assays of relative activities, and for high throughput applications need only be done once. This SPA method can be used in time course assays for kinetic measurements, for quantitation of methyltransferases and for assay of inhibitors in DMSO concentrations up to 15% by volume. It is easily adapted to HTS processes and for manual procedures offers the advantages over the filter binding assay of less time, materials, radioactive waste and lower overall cost.

ACKNOWLEDGEMENTS

We thank Dr Heather C. O’Farrell for providing purified KsgA and wild-type and ksgR strains of E. coli, Dr Aiye Liang for her help with Sigma Plot, and Dr Gordona Maravic Vlahovic for providing us with a plasmid containing the ErmC gene.

FUNDING

This work was funded from grants from the Commonwealth Health Research Board (to J.P.R. and H.T.W.) and the National Institutes of Health (to J.P.R., GM66900). Funding for open access charge: National Institutes of Health.

Conflict of interest statement. None declared.

REFERENCES


