Supplemental Materials

Optimization of DBCO-biotin labeling

The original protocol by Flynn *et al.*[S1] involves the DBCO-biotin reaction at 55°C for 10 minutes in the presence of the dye-free loading buffer. Additionally, the DBCO-biotin reaction occurs after RNAs are digested with proteinase K and purified.

Several reasons motivated us to test additional conditions. First, we occasionally observed some levels of RNA degradation after the DBCO-biotin reaction. Because RNAs are less stable at higher temperatures, we questioned whether lowering the reaction temperature is feasible. Second, 10 minutes of the reaction duration means small differences of incubation time (e.g. one minute too long for some samples) may lead to variations in the final glycoRNA signals if the reaction is not close to completion. Third, RNAs need to go through protein K digestion followed by purification and then by DBCO-labeling and then another round of RNA purification. We questioned whether it is feasible to have a single step reaction to not only save on time and reagents, but also reduce the RNA loss as the consequence of an extra step of purification. Fourth, we questioned whether it is necessary to react in the presence of the dye-free loading buffer. Fifth, we questioned whether putting samples on ice can effectively stop the reaction.

We show some of the optimization results below. We found that reacting at 37°C for 30 minutes yields consistently better signals than 55°C for 10 minutes, and we never observed RNA degradation with the reaction at 37°C for 30 minutes. We found consistently equal or slightly better signals when reaction is conducted in water without the dye-free loading buffer. Additionally, increasing DBCO-biotin concentration can further enhance signals. And finally, a one-step reaction is feasible, albeit at slightly reduced reaction efficiency compared to the two-step protocol. We have also found that reaction on ice for 30 minutes does not yield detectable signals, whereas the reaction in room temperature (~20°C) is much slower than the reaction at 37°C (results not shown). Therefore, one can stop the reaction by returning tubes to ice, but leaving the reaction in room temperature is not recommended.

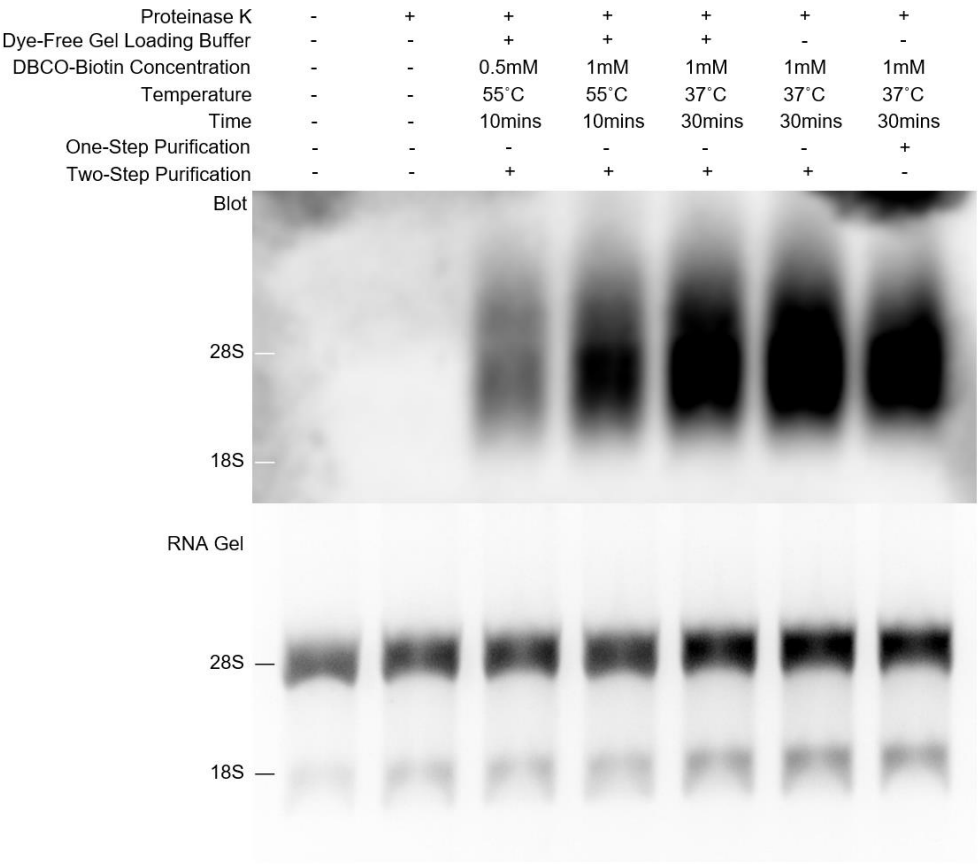

Figure S1. Optimized DBCO-Biotin Labeling Condition.

Total RNAs were extracted from Ac4ManNAz-treated Ba/F3 cells. RNAs were then subjected to proteinase K digestion and DBCO-biotin labeling as indicated. Most samples underwent the two-step protocol as indicted, whereas the last lane shows the sample that used the one-step protocol. The different reaction temperatures, time, concentrations of DBCO-biotin, and whether the dye-free loading buffer is included are indicated. In reactions containing the dye-free loading buffer, the volume of the dye-free loading buffer is 50% of the reaction volume. Reactions were stopped on ice before purification. The top panel shows the SA-HRP signals, whereas the bottom panel shows the RNA bands on the gel before transfer. The positions of 28S and 18S rRNAs are labeled.

Optimization of RNA Dye Concentration

The RNA signal on the denaturing gel is crucial for the interpretation of sample equal loading and for the normalization of the glycan signal on the northwestern blot when comparing different experimental groups. We have modified the RNA dye concentration from Flynn *et al.*,[S1] based on the representative data shown in **Figure S2**. We evaluated SYBR Gold, SYBR Safe, GelStar, and Diamond nucleic acid dyes. We first tested different concentrations of SYBR Gold and Diamond nucleic acid dyes. We found that at the manufacturer recommended dilution of 1:10,000 (or sometimes referred to as 1X), neither of the dyes can detect differential RNA quantities, making RNA amounts as different as 8-fold (ranging from 1.25µg to 10µg) appear with similar band intensities (**Figure S2A, S2B**). Among these four dyes, when used at a 1:100

concentration, we found that SYBR Gold and Diamond outperformed SYBR Safe and GelStar, judged by their ability to report differential RNA amounts and sensitivity (**Figure S2C**). The SYBR Gold and the Diamond Nucleic Acid Dye performed similarly in reporting RNA amounts and when glycoRNA signals do not overlap the rRNA bands. When the glycoRNA signals overlap with the 28S rRNA band, as in the example shown in **Figure S2D**, both dyes reduced the signal at the position of the 28S rRNA band compared to the no-dye control. SYBR Gold is favored over the Diamond Nucleic Acid Dye as the effect of the dye on the overlapping glycoRNA signal is relatively minor.

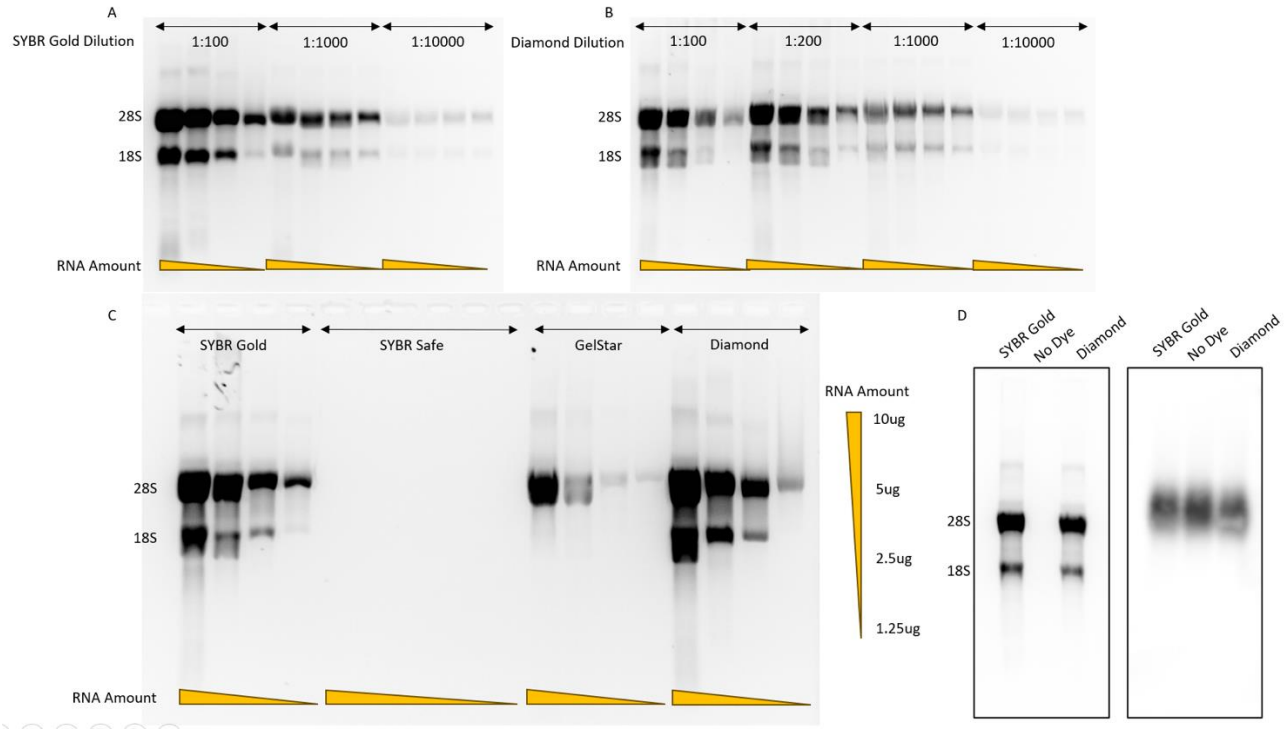

Figure S2. Optimized nucleic acid dye type and concentration for RNA denaturing gel electrophoresis.
(A) Total RNAs were loaded with 1:100, 1:1000, or 1:10000 of SYBR Gold Nucleic Acid Dye in a total volume of 20µL per lane for electrophoresis. 10µg, 5µg, 2.5µg, or 1.25µg of RNAs from each SYBR Gold dilution group were loaded onto a denaturing formaldehyde gel. The gel was imaged after electrophoresis.
(B) Total RNAs were loaded with 1:100, 1:1000, or 1:10000 of Diamond Nucleic Acid Dye in a total volume of 20µL per lane. 10µg, 5µg, 2.5µg, or 1.25µg of RNAs from each Diamond dilution group were loaded onto a denaturing formaldehyde gel. The gel was imaged after electrophoresis.
(C) Total RNAs were loaded with SYBR Gold, SYBR Safe, GelStar, or Diamond Nucleic Acid Dye. 10µg, 5µg, 2.5µg, or 1.25µg of RNAs from each group were loaded onto a denaturing formaldehyde. The two wells in the middle of SYBR Safe were not loaded with RNA due to bubbles inside the well. All dyes were used at 1:100 final concentration in a total volume of 20µL per lane. The gel was imaged after electrophoresis.
(D) Left panel: Total RNAs were prepared from K562 cells treated with Ac_4ManNAz and reacted with DBCO-biotin via the one-step protocol. RNAs (5µg) were loaded onto the gel with 1:100 SYBR Gold, no dye, and 1:100 Diamond Dye. The gel was imaged after electrophoresis. Right panel: Northwestern blot was performed, and glycan signals were visualized. Shadows created by the dyes were observed at the position

of 28S rRNA of both the SYBR Gold and Diamond dye lanes, with shadow in the Diamond dye group being more prominent.

Optimization of Northwestern Blot Blocking Condition

We have modified the blocking and staining conditions from Flynn *et al.*[S1] based on data shown in **Figure S3**. We tested 5% milk, Bio-Rad EveryBlot blocking buffer, and Li-Cor Intercept (PBS) blocking buffer. We found that when using SA-HRP as a detector, blocking and staining the nitrocellulose membrane in the Bio-Rad EveryBlot blocking buffer outperformed the other two substantially with a lower background, as it allows for a detection of weak glycan signals (e.g. from a low amount of RNA) on the nitrocellulose membrane.

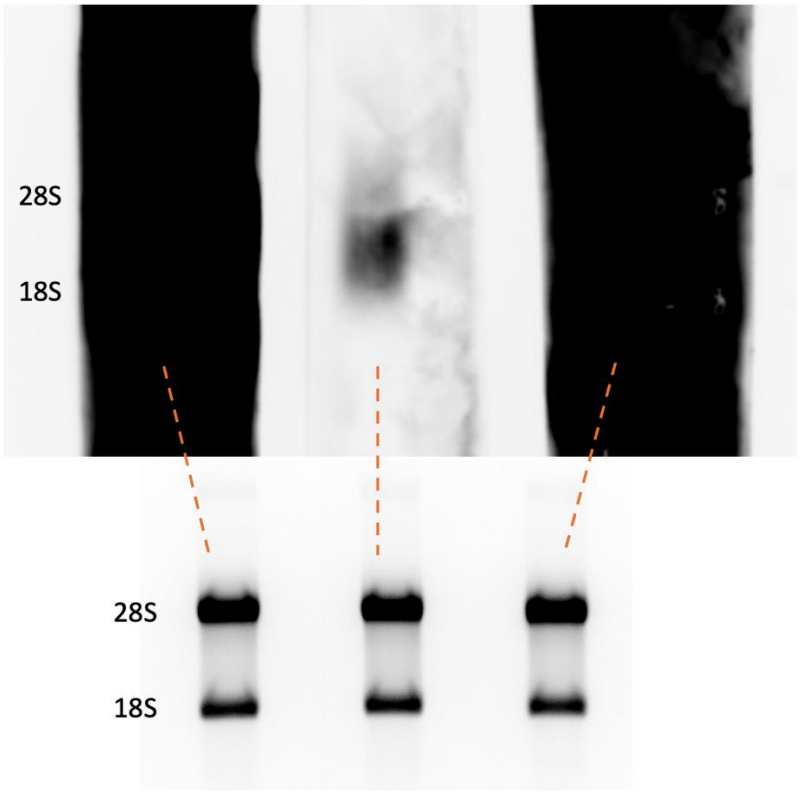

Figure S3. Optimization of the blocking condition for northwestern blots

Top panels: Total RNAs were extracted from Ac4ManNAz-treated Ba/F3 cells, treated with proteinase K, and DBCO-labeled. Three types of blocking buffers were tested: 5% milk, BioRad Everyblot blocking buffer, and Intercept blocking buffer (from left to right in the figure). 1.4µg of RNAs were loaded onto the denaturing formaldehyde gel per lane. For the BioRad Everyblot blocking buffer and the Intercept blocking buffer groups, after blocking, we removed blocking buffer, and stain the blots by adding 1:5000 of SA-HRP into the corresponding blocking buffers used. For the 5% milk group, after using milk to block, we washed the blot three times with 1X TBST, and stained the blots by adding 1:5000 of SA-HRP into the Intercept

blocking buffer. The positions of 28S and 18S rRNAs are indicated. Bottom panel: gel image of each of the three lanes.

Supplemental References

[S1] R. A. Flynn *et al.*, "Small RNAs are modified with N-glycans and displayed on the surface of living cells," (in eng), *Cell,* vol. 184, no. 12, pp. 3109-3124.e22, Jun 10 2021, doi: 10.1016/j.cell.2021.04.023.