Development of novel luminescent assays for sensitive and specific quantitation of double-stranded RNA in mRNA therapeutics

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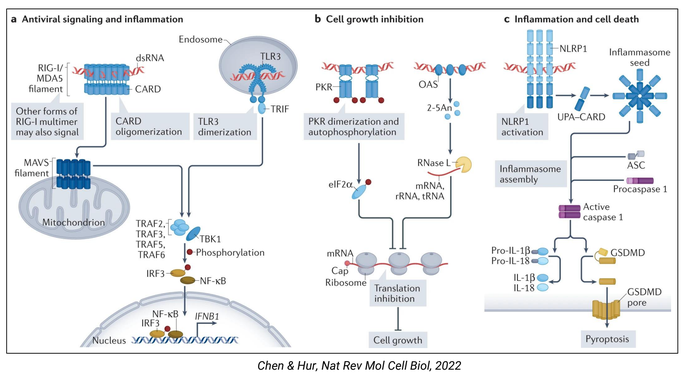
1. Introduction

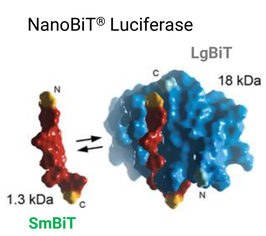
- Double-stranded RNA (dsRNA) is a byproduct and contaminant of in vitro transcription (IVT) products
- dsRNA is highly immunogenic and can be detected by several intracellular or endosomal sensors, leading to inflammation, translation inhibition, and cell death



Chen & Hur, Nat Rev Mol Cell Biol, 2022

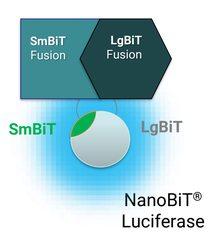
- Existing methods to detect dsRNA in mixed solutions lack accurate quantitation, specificity, and sensitivity
- **We have developed novel assay systems for dsRNA detection using bioluminescence**
  - Lumit® dsRNA Detection Assay using NanoBiT® technology
  - dsRNA detection using cell-based TLR3 reporter bioassay

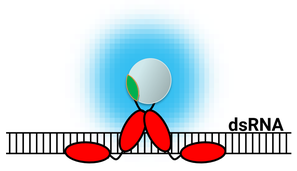
2. dsRNA detection using Nanoluciferase Binary Technology (NanoBiT®)



- Nanoluciferase is split into two subunits: SmBiT and LgBiT
- Complementation of SmBiT and LgBiT generates a functional luciferase and the generation of light in the presence of substrate
- SmBiT and LgBiT have very low affinity in solution

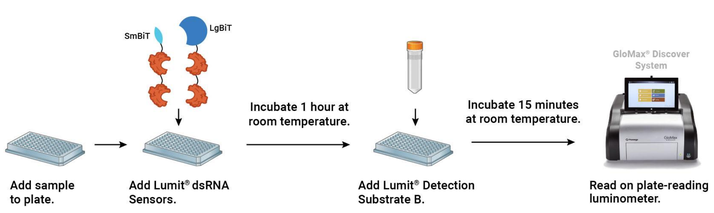
- Forced complementation of the NanoBiT® luciferase is achieved by fusion of the BiTs to binding partners
- Interaction of the binding partners brings the SmBiT and LgBiT into proximity, complementing the luciferase





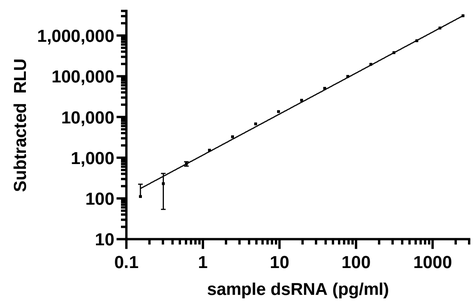
- SmBiT and LgBiT are genetically fused to dsRNA binding domains (DRBDs, red)
- Dimerization of the BiT-DRBDs on dsRNA (>30bp) induces complementation of NanoBiT® luciferase and generation of light

3. Lumit® dsRNA Detection Assay protocol



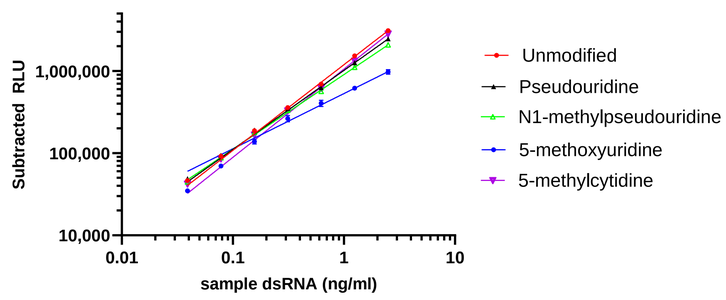
- Solution-based homogenous assay
- Simple add-mix-read format
- No washing or sample transfer steps
- Easily scalable and amenable to automation

4. Sensitive detection of dsRNA



- **Limit of Detection**: 0.37 pg/ml
- **Limit of Quantitation**: 1.12 pg/ml
- **Linear Range**: 0.04ng/ml - 2.5ng/ml

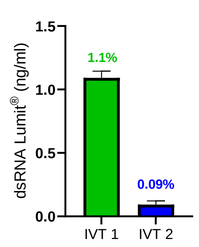
5. Detection of dsRNA containing nucleotide modifications



- Each dsRNA standard was HPLC-purified to equivalent purity and tested in three independent experiments
- The assay demonstrated linear detection ranges across all standards tested

6. Specific detection of dsRNA in IVT products

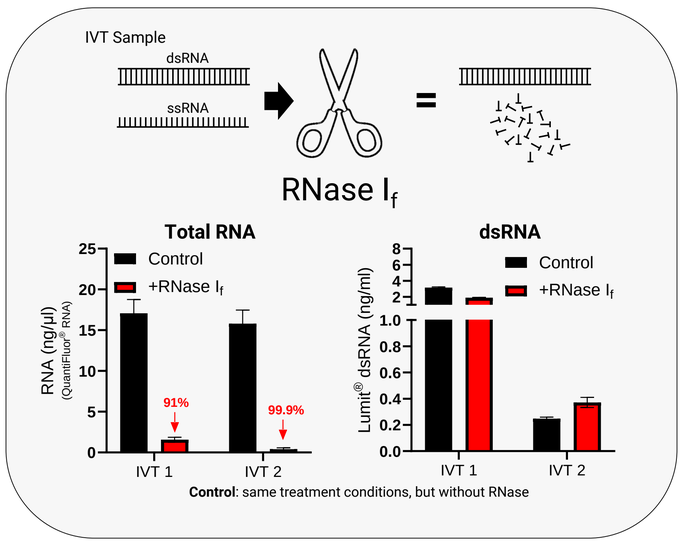
Quantitation of dsRNA in IVT products

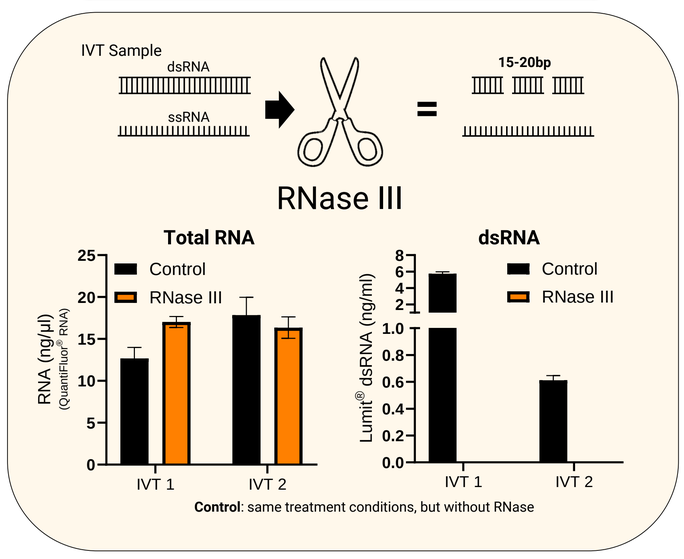


- The Lumit® dsRNA Detection Assay was performed with two IVT products as samples
  - IVT 1: purified with $LiCl_2$
  - IVT 2: DEAE column-purified
- Percentage shown is dsRNA / total RNA

Specific detection of dsRNA in mixed samples

- IVT samples were treated ± RNase I_f (ssRNA-specific) or RNase III (dsRNA-specific), total RNA (QuantiFluor® RNA) and dsRNA (Lumit®) were quantified
  - **Top:** Digestion of ssRNA significantly reduced total RNA, but did not impact the amount of dsRNA detected
  - **Bottom:** Digestion of dsRNA into fragments ≤ 20bp results in a complete loss of activity in the Lumit® dsRNA Detection Assay

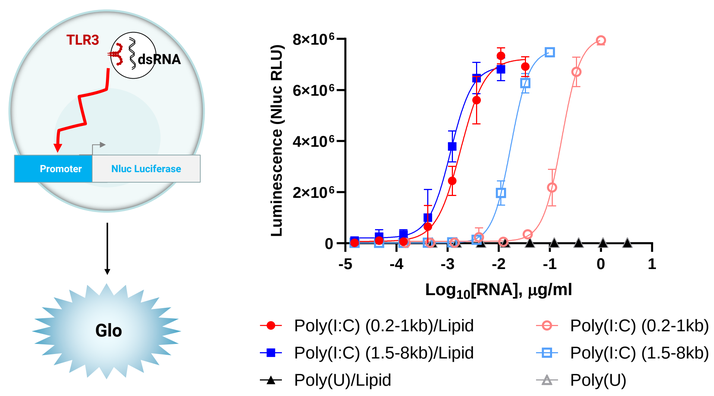




7. Specific detection of dsRNA by the TLR3 Bioassay

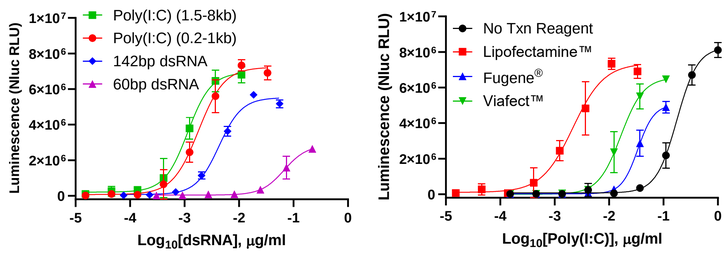
Assay design

1. dsRNA is taken up by the reporter cell
2. Binding of dsRNA to TLR3 in the endosome
3. Activation of TLR3-responsive promoter drives luciferase expression and light output

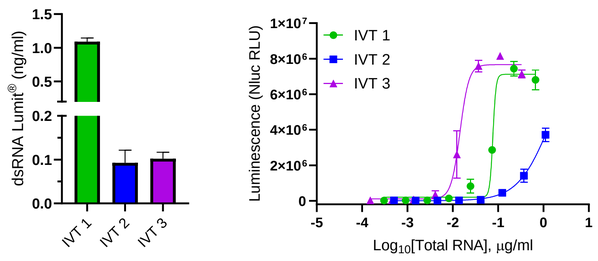


- Poly(I:C) or Poly(U) with or without lipid transfection reagent (Lipofectamine™ RNAiMAX) was tested in the TLR3 Bioassay
- No response to ssRNA (Poly(U)) was observed

8. Differences in TLR3 activation by dsRNA size and sequence



- The TLR3 Bioassay was performed with Lipofectamine™ RNAiMAX-complexed dsRNA samples of different size and sequence (left) or with Poly(I:C) (0.2-1kb) with different lipid transfection reagents (right)
- **The TLR3 Bioassay response is dependent on dsRNA size and/or sequence as well as RNA delivery method**



- The Lumit® dsRNA Detection Assay (left) and the TLR3 Bioassay (right) were performed with three IVT products as samples
  - IVT 1: purified with $LiCl_2$ (0.4kb product)
  - IVT 2: DEAE column-purified (1.8kb)
  - IVT 3: DEAE column-purified (1.2kb)
- For the TLR3 Bioassay, samples were complexed with Lipofectamine™ RNAiMAX
- **TLR3 Bioassay response is likely dependent on size and/or sequence of dsRNA contaminants, not necessarily total amount of dsRNA**

9. Conclusions

We have developed two new tools for dsRNA detection and quantitation

- The Lumit® dsRNA Detection Assay provides sensitive quantitation of dsRNA in mixed solutions, and overcomes the inadequacies of existing ELISA and dot blot technology
- Cell-based reporter bioassay enables MoA-relevant measure of cellular response to dsRNA in encapsulated mRNA preparations

Easy-to-implement

- Rapid and convenient workflows
- Lumit® dsRNA Detection Assay features a simple add-mix-read protocol with no immobilization or wash steps
- TLR3 Bioassay can be used in "Thaw-and-Use" format, no cell culture required

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