A guide to *In Vitro* Transcription (IVT) and the importance of dsRNA detection

Introduction

In vitro transcription (IVT) is a molecular biology technique that allows for the synthesis of RNA molecules based upon a chosen DNA template in a biochemical format. It is a basic yet key technique for many research areas and biotechnological/pharmaceutical applications because of how simple it is to implement, how precise, versatile and effective it is at generating RNA transcripts, and how rapidly scalable of a method it is.

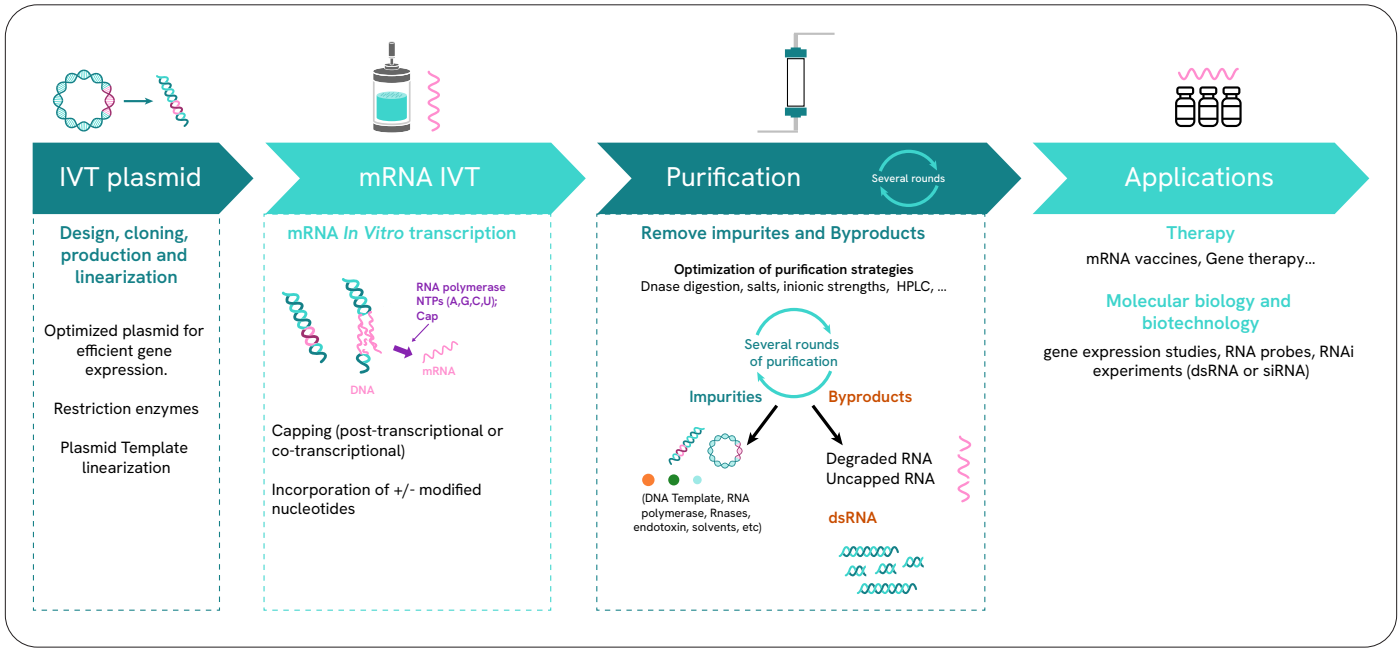

Figure 1: Global process of *In Vitro* Transcription.

In vitro transcription is run in several steps:

1. **Template** - The DNA template to be transcribed into ssRNA need to be selected and edited to include a promoter region that makes it eligible for RNA polymerase binding. The most common promoters used are typically those of T7, T3 or SP6 polymerases. The DNA template can be plasmidic or linearized. While plasmids are more stable, linearized sequences promote more efficient transcription.

2. **Setup and transcription** – the *in vitro* transcription mix contains the DNA template, an RNA polymerase, ribonucleotide triphosphates, and appropriate buffers and cofactors. Once put together, the mix is incubated. While the temperature and duration of the incubation can be adapted to control the reaction, standard protocols recommend 1-2 hours at 37 °C. During this reaction, the polymerase binds the DNA template on the corresponding promoter and synthesizes the matching ssRNA by adding nucleotide triphosphates in a strand complementary to the template.

3. **Purification –** Post-transcription, the IVT result contains the intended ssRNA sequence, as well as the DNA template, polymerase, leftover nucleotides, and contaminants, including dsRNA. Common methods of removing undesired components include phenol-chloroform extraction, column-based purification, and ethanol precipitation.

4. **Characterization –** The resulting ssRNA is characterized to validate its titration, integrity and fidelity to the template. Several techniques can be used, including qRT-PCR, spectrophotometry, and gel electrophoresis.

In terms of application, IVT has many uses in several areas of molecular biology and biotherapeutic research: RNA probes for hybridization assays, RNA interference experiments (gene therapy applications), gene expression studies, CAR T-cell editing, etc. Most importantly, IVT plays a significant role in synthetic biology and therapeutics. It was pivotal in the development of RNA-based therapeutics like mRNA vaccines for COVID-19 and RNA-based drugs. In these particular settings, IVT allows for the controlled and mass production of target RNA sequences from a DNA template of interest. These applications -especially mRNA vaccines - have exploded in the wake of the COVID-19 pandemics, when the outpour of resources into dedicated research advanced techniques development very quickly and allowed for the effectiveness of mRNA vaccines to be successfully demonstrated globally. Even more so than for other applications, RNA-based therapeutics call for rigorous and robust QA/QC methods as they ultimately aim to receive approval from regulation authorities and are destined for patient use directly.

Understanding dsRNA contaminants in IVT Samples

dsRNA or double-stranded RNA is a nucleic acid molecule that is made of two strands of single-stranded RNA (ssRNA), intertwined together in a double helix structure much like DNA. Unlike DNA however, it usually has an alpha-helix form due to the ribose groups it carries.

There can be multiple reasons why dsRNA is found as a contaminant in IVT products, the most common ones being cases of secondary structures from ssRNA, self-annealing, random annealing of complementary short abortive transcript, or RNA polymerase pausing and DNA template contamination.

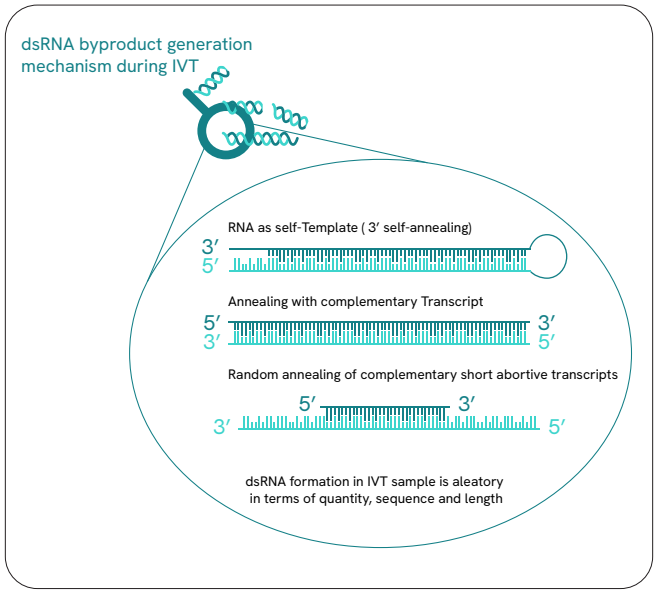

Figure 2: Causes for dsRNA contaminants in IVT processes.

The significance of dsRNA contamination in IVT processes is massive and cannot be ignored because, while it is unavoidable, it also causes potential risk with the end product of the transcription. dsRNA is normally entirely absent from our cells, as our DNA replication/transcription/expression process does not rely on a dsRNA step at any point. In normal circumstances, the only time dsRNA is present in human cells is when it is being brought from the outside by a foreign entity, typically a virus. For that reason, the innate part of our immune system has developed an array of nucleic acid receptors and inflammatory pathways that are capable of picking up on the presence of dsRNA in the cytosol and triggering inflammatory responses to deal with and destroy what is supposed to be a threat. The main receptors playing these roles and being at risk of being activated are RIG1/MDA5 and TLR3, which all have ramifications with potent inflammatory signaling cascades and apoptosis pathways such as the caspase cascade.

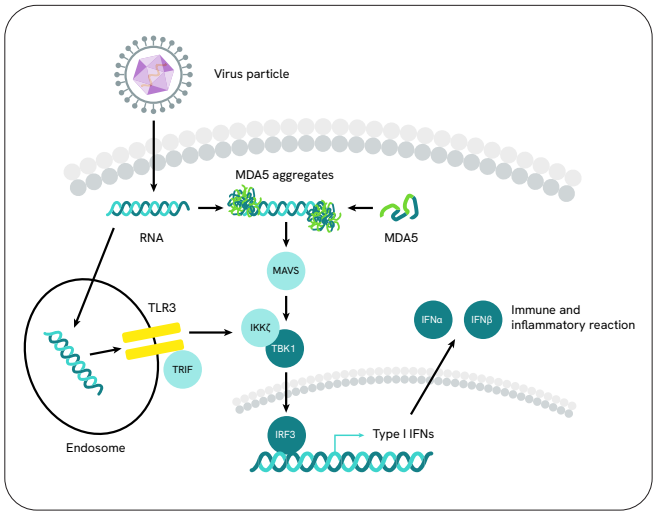

Figure 3: Innate immunity pathways and mechanisms that detect dsRNA.

For this reason, dsRNA carries a strong potential for immunogenicity and adverse effects when injected into patients and must be monitored and removed from biotherapeutics to avoid such complications.

Introduction to the challenges of quantitative dsRNA detection methods

dsRNA detection antibodies

There are several antibody clones that are typically used for dsRNA detection. Among those, a handful have been tested, validated and referenced across literature sufficiently to be considered as gold standards for their intended purpose (Schönborn et al., 1991; We et al, 2020).

The clones most commonly considered as gold standards are J2, which is the most widely used clone, K1, K2, and 9D5.

They all differ by their affinities and binding properties, but share the following set of characteristics:

- Varying affinity for different sequences dsRNA. This is caused by dsRNA fragments being more or less flexible and condensed based on their sequence, which renders the repeat epitopes less accessible for antibodies. In particular, the G-C sequence confers higher flexibility than the poly-G sequence. Generally, these antibodies are described to display higher affinities for synthetic dsRNAs. They will bind to natural and synthetic dsRNA, but with different affinities.
- Minimal dsRNA length detected is described as 40 bp. These clones are also described to have different affinities depending on the fragment size due to the repeat epitopes of helical structures they target.

Most ELISA and Dot-blot assays currently in use rely on at least one of these clones, and as such, it is generally recommended to use them to design or develop new immunoassays that aim to improve upon current techniques.

About dsRNA fragment length

Immediately after transcription, IVT products contain contaminant dsRNA fragments of various sizes in random quantities. The size of these fragments is important and must be taken into account when selecting a quantification method for two reasons.

Firstly, the antibodies available for dsRNA detection target repeat epitopes in the nucleic acid sequence, which makes them bind to longer sequences more. A 1000 bp fragment would theoretically bind 10 times more detection antibodies than a 100 bp one of similar composition and sequence, resulting in stronger signal. That difference can be a cause for bias as the composition of IVT samples is not necessarily homogeneous with short and long sequences being present in unequal amounts.

Secondly, dsRNA fragments tend to be rather small and range from 50-250 bp for the most part, with outliers going as low as 20-30 bp. This poses challenges for detection that must be clear to users as they establish QA/QC processes to test their IVT samples.

Impact of standards on dsRNA detection

Due to the very heterogeneous nature of IVT mixes in terms of dsRNA fragment lengths, the standard used for dsRNA quantification is key, regardless of the chosen detection method. Ideally, the standard composition should be as close as possible to the IVT product to ensure the detection antibody epitopes are present in relatively equal amounts. If the standard is very different from the sample, the assay runs the risk of being biased by the standard when quantifying dsRNA in the sample.

dsRNA detection methods

There are currently three main immunoassay formats that are commonly used to detect and/or quantify dsRNA in IVT samples.

- The ELISA is the most widespread one and is largely available and/or customizable using different gold standard antibodies referenced in the literature. It allows both the detection and quantification of dsRNA.

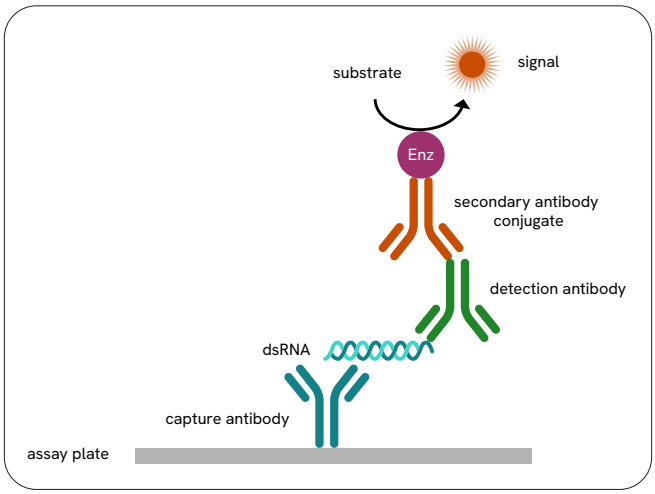

Figure 4: ELISA assay principle.

- The Dot-blot is the other traditional approach used in the industry, which is very similar in principle to the Western blot. Instead of being separated via electrophoresis, the samples and loaded and blocked onto a membrane in a circular template (or dots), before being treated with detection reagents that light up dots containing the analyte of interest.

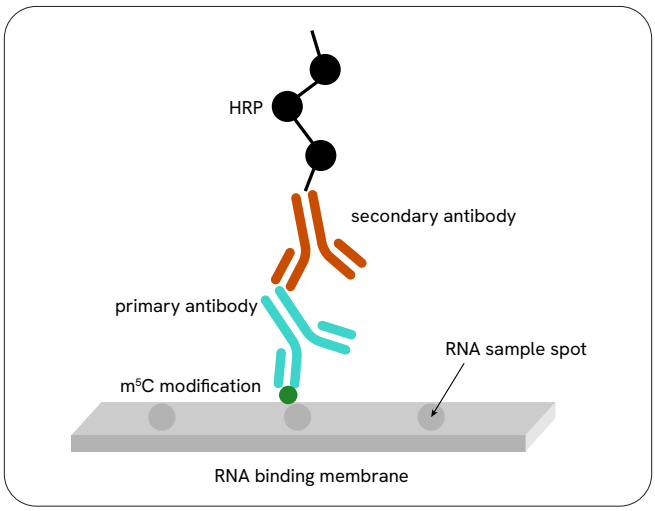

Figure 5: Dot-blot assay principle

- Homogeneous immunoassays, like the HTRF dsRNA (IVT) Assay developed by Revvity, relies on FRET signaling where two detection antibodies labeled with donor and acceptor fluorophores are introduced to the IVT samples and lit with a specific wavelength of light. The donor fluorophore absorbs that wavelength and emits back another that can be picked up by the acceptor fluorophore and re-emitted as a third wavelength. That energy transfer between the two fluorophores is called FRET signaling and only occurs when both are in very close proximity, i.e., bound to the same molecule by the antibodies that carry them. The resulting signal is proportional to the amount of antibodies bound to their target, and therefore, to the concentration of target in the sample.

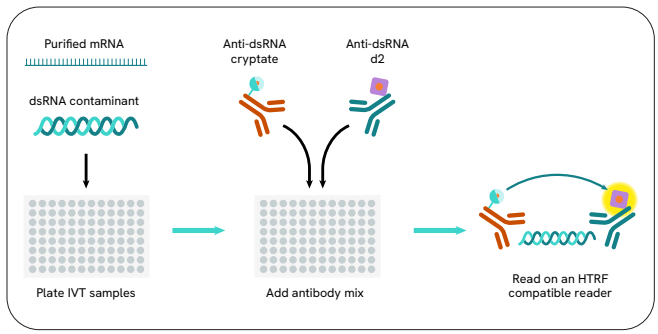

Figure 6: HTRF dsRNA (IVT) assay principle

This assay was developed with IVT applications specifically in mind and incorporates a number of differences with regularly available ELISA or Dot-blots. It also offers improvement upon the other Revvity HTRF dsRNA (virology) assay, whose characteristics are more oriented toward applications in viral contamination detection.

- The standard used is one of natural dsRNA to be closer in composition to IVT samples. The point is to improve upon synthetic Poly-IC standards that are commonly used in dsRNA immunoassays (ELISA and Dot-blot) that introduce bias due to their inherent difference of composition with the sample, regardless of sequence. (See Page 9 – Fig. 13 for experimental data).
- The antibody mix used for the detection includes a gold standard antibody as referenced in Schönborn *et al.*, 1991; We *et al*, 2020 chosen to give the most sensitive assay.

- The incubation temperature of the assay was also taken into account in this development. With dsRNA detection and quantification being a QA/QC step that is ideally run at several stages of production and purification, it is time-effective to have an assay capable of running at room temperature. An even better sensitivity and signal can be achieved by incubating and running the assay at 4 °C.

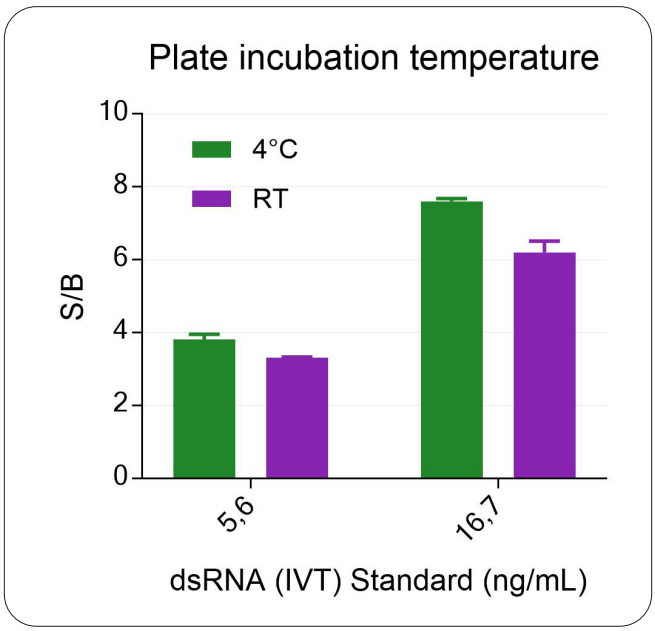

Figure 7: Comparative detection of dsRNA with the HTRF dsRNA (IVT) Assay at 4°C and room temperature.

- As mentioned previously, the length of dsRNA fragments that can be detected is sought-after information for IVT users. Consequently, the HTRF dsRNA (ITV) Assay is thoroughly tested to detect fragments in the lower range of sizes, with a reliable detection of 50 bp fragments at low concentrations and as low as 30 bp for high concentrations (See Page 6 - Fig. 9 for experimental data).
- The incubation time of the assay is rather flexible, allowing for reading after as little as 1 hour of incubation, which supports routine use in parallel with purifications steps. Although these results are accessible from 1 hour, it is also possible to improve them marginally by increasing the incubation time. The maximum is thus obtained after overnight incubation.

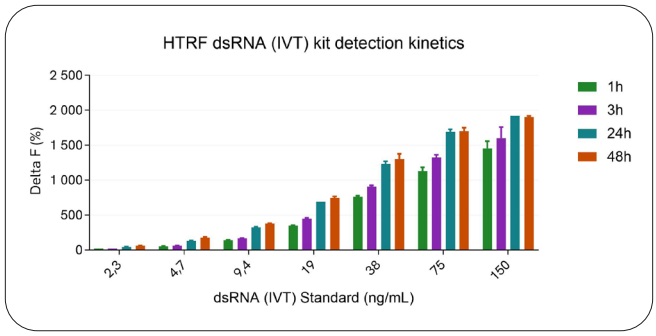

Figure 8: Detection of dsRNA using the HTRF dsRNA (IVT) Assay after different incubation times. About 70% of equilibrium is reached after 1 hour incubation. Full equilibrium is reached after overnight incubation.

Case studies/validation data

Length of dsRNA fragments in IVT samples

Post-IVT samples contain dsRNA contaminants of various sizes, with the most prevalent sequence sizes ranging from 50 bp to 250 bp. Given that measuring the dsRNA content is a critical quality control element of IVT, we sought to precisely determine the kit's capability to detect dsRNA sizes across this range and beyond.

The samples used in the following experiments are bulks of dsRNA contaminants present in IVT products after transcription. All samples were serially diluted in the kit diluent, and 10 µL of each dilution was plated in a 384sv white plate. After addition of 10 µL of HTRF dsRNA (IVT) detection reagents mix, the HTRF signal was recorded following overnight incubation at room temperature.

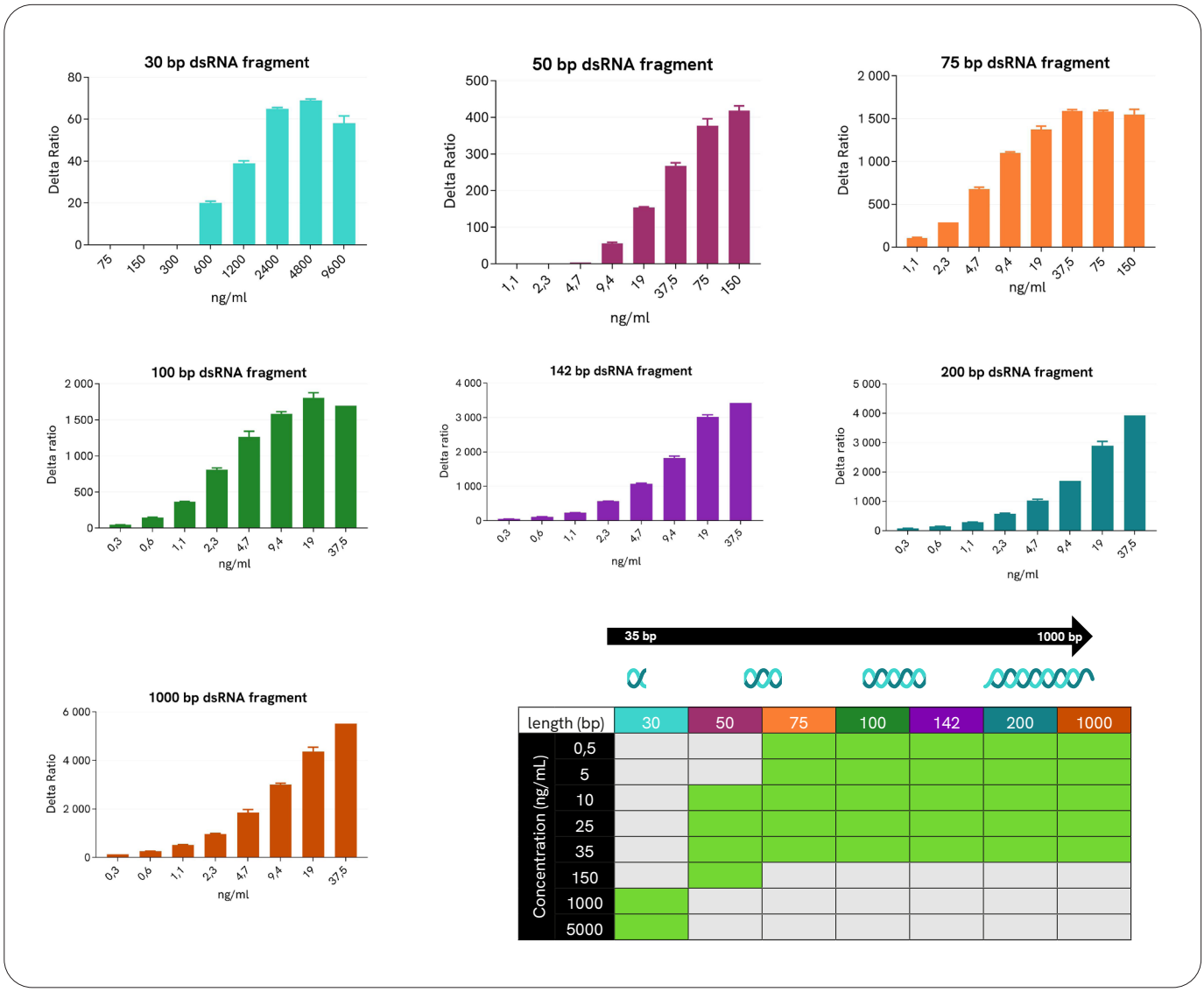

Figure 9: Detection of dsRNA sequences of 30, 50, 100, 142, 200 and 1000 bp with the HTRF dsRNA (IVT) assay.

Detection of dsRNA was successful for the whole range of short and mid-length sequences. In particular, we noticed that the 50 bp sequences (lower end of the usual range of sequences in IVT products) were detected at low concentrations. The detection of 30 bp sequences was possible, but required a higher concentration of such sequences in the sample. It was also noticeable that the sensitivity of the detection was enhanced with longer dsRNA sequences.

Detection was successful with sequences far above the IVT range. Here we show that a 1000 bp sequence is detected even at low concentrations.

Very short sequences – 20 bp

The experiment with very short sequences of 20 bp was performed with additional controls to confirm what the assay actually detects. The assay was run side-by-side on three different sequences:

- A 20 bp sequence generated in the same way as the samples in previous experiments.
- A 100 bp sequence made of 5 of the 20 bp sequences put together. This serves to verify whether a possible lack of detection at 20 bp is the consequence of the sequence lacking the target epitope (in which case we also would not see detection of the 100 bp sequence), or if it is because it is impossible for the antibodies to bind a sequence under a certain size, even if that sequence carries the target epitopes.

- A wild type 100 bp sequence that serves as a control to confirm the assay detects a 100 bp sequence in this experiment and validates the use of the other control.

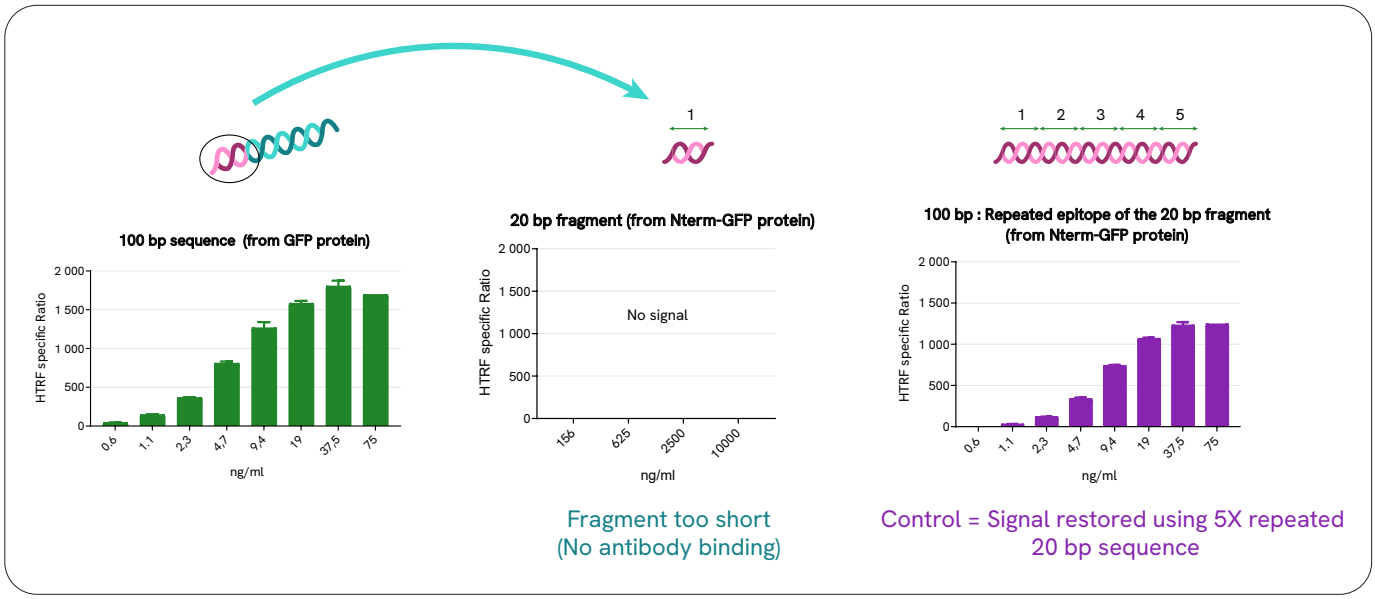

Figure 10: Detection of dsRNA sequences of 20 bp with the HTRF dsRNA (IVT) assay.

The results show a lack of detection for the 20 bp sequence. However, both 100 bp sequences are detected, which confirms the assay does not detect these very short sequences not because they lack the target epitopes, but because their size does not allow for the binding of the detection reagents.

We notice a 25-40% difference in signal between the wild type and repeat epitope 100 bp sequences, with the wild type one getting better results. This is expected as the two sequences are different, and therefore, bind the antibodies in slightly different proportions.

Conclusion

- Detection is successful from 50 bp to 1000 bp at low concentrations, which covers the usual range of dsRNA contaminants in IVT products.
- Detection is possible at 30 bp for higher concentrations.
- Detection is improved with longer dsRNA sequences.
- Detection is not possible at 20 bp and below.

These results are expected due to the repeat epitope-based detection of the assay. Longer sequences are more susceptible to binding the fluorophore-carrying antibodies, which results in brighter signals compared to shorter sequences. The results also indicate that the lack of detection of very short sequences is due to the dsRNA length not allowing binding and not because of a lack of epitope on the sequence itself. This latter finding could mean that other detection methods involving antibodies or dsRNA-binding proteins may suffer from the same limitation, where some dsRNA are simply too short to be targeted.

Detection of uridine-modified dsRNA

The use of modified nucleoside triphosphates (NTPs) has been instrumental in advancing mRNA therapeutic innovation. In particular, N1-metylpseudo-uridine has become one of the most widely used and effective base modifications in mRNA therapeutics for three reasons:

Pseudo-uridine replaces uridine (U) in the mRNA sequence, leading to more accurate and efficient synthesis and improved **fidelity of mRNA transcription.** Pseudo-uridine also enhances **RNA stability**, making the mRNA less susceptible to degradation. This stability is important for maintaining the

integrity of mRNA therapeutics and vaccines during storage, transportation, and administration. Finally, pseudo-uridine incorporation **decreases the anti-RNA immune response**. mRNA modified in this way is less likely to assemble in immunogenic dsRNA, and pseudo-uridylated mRNA is better tolerated by the immune system.

In this experimental setup, two 100 bp dsRNA sequences were synthetized from the N-terminus portion of GFP protein. One sequence was generated without modification, while the other had a substitution of all uridines by **N1mePseudo-UTP**. The HTRF dsRNA (IVT) assay was run following the protocol and compared to a Dot-blot assay using the J2 gold standard antibody.

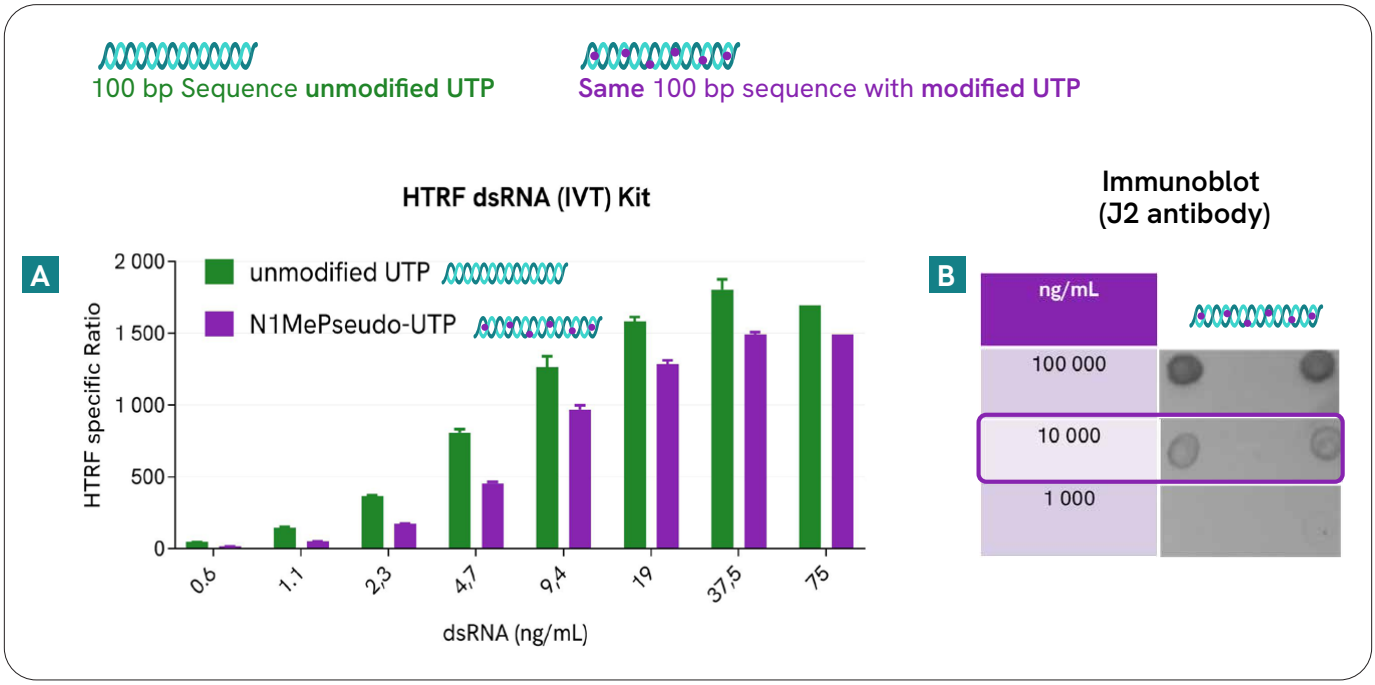

Figure 11: A: Comparative detection and quantification of uridine-modified and unmodified dsRNA with the HTRF dsRNA (IVT) assay. B: Detection of uridine-modified dsRNA with a J2-antibody immunoblot assay.

Table 1: Limit of quantification of HTRF dsRNA (IVT) assay and J2-antibody immunoblot assay.

Limit of quantification of modified UP dsRNA	
HTRF dsRNA (IVT) kit	**Dot-Blot (J2)**
1 ng/mL	10 000 ng/mL

The resulting HTRF signal and dot fluorescence intensity showed that both assays are capable of detecting N1mePseudo-UTP dsRNA. In the case of the HTRF assay, sensitivity for modified dsRNA was in the same range as that of unmodified dRNA, with a similar detection limit around 1 ng/mL. For the immunoblot assay, however, the detection limit was much higher and around 10000 ng/mL.

Example of application to viral material with COVID-vaccine antigenic sequence

With dsRNA detection and quantification in IVT products being an especially important element in the development and production of mRNA vaccines, we sought to evaluate the relevance of the assay in the context of a viral sequence. Here we present an experiment with a dsRNA sample consisting of a 100 bp viral sequence coding for the Spike protein of SARS-CoV-2 that is used in the mRNA vaccine developed and commercialized by BioNTech and Pfizer. The exact reference of the sequence is found at CDD Conserved Protein Domain Family: SARS-CoV-2_Spike_S1_RBD (nih.gov).

The HTRF assay was run side-by-side an immunoblot assay.

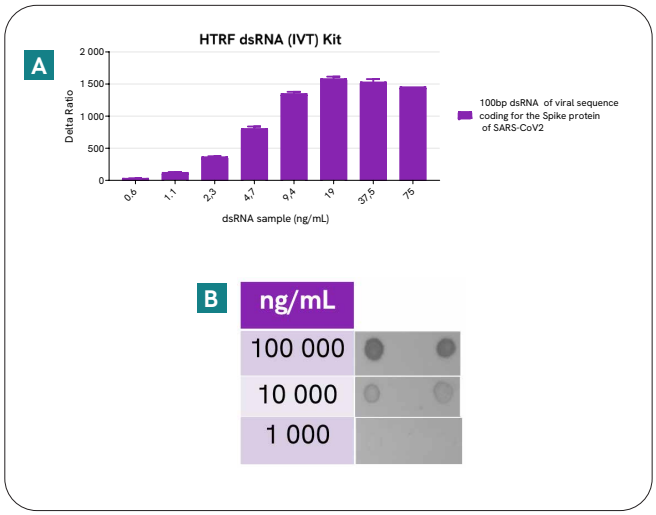

Figure 12: A : HTRF detection of a 100bp dsRNA from SARS-CoV2 vaccines. B: Immunoblot detection of a 100 bp dsRNA from SARS-CoV-2 vaccines.

Table 2: Limit of quantification of HTRF dsRNA (IVT) assay and J2-antibody immunoblot assay from SARS-CoV2 vaccines.

Limit of quantification (100 bp dsRNA from spike protein sequence of SARS-coV2 virus)	
HTRF dsRNA (IVT) kit	**Dot-Blot (J2)**
1 ng/mL	10 000 ng/mL

The results indicated that both the HTRF kit and immunoblot assay allowed for the detection of that SARS-CoV-2 sequence, with the HTRF assay having significantly better sensitivity.

Effects of standard on dsRNA detection

We present here standard curves using the natural dsRNA standard from the HTRF dsRNA (IVT) Kit and a synthetic Poly-IC standard.

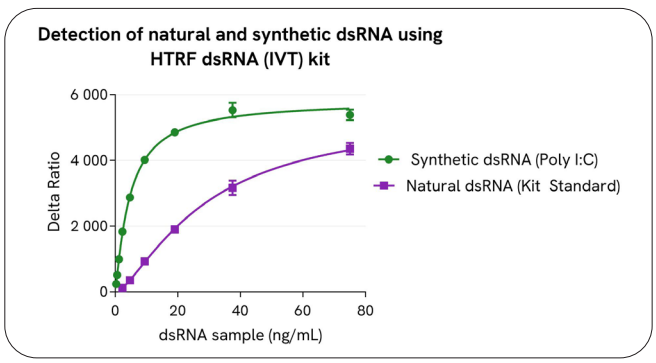

Figure 13: Standard curves of natural and synthetic Poly-IC dsRNA standards

The results reported a difference of 4-fold in dsRNA sample quantification between both standards. Due to that bias coming from the synthetic Poly-IC standard, dsRNA sample concentration was underestimated in that case.

This difference was expected and reported in the literature as a consequence of different antibody affinities for natural and synthetic dsRNAs. This has been well-documented for anti-dsRNA gold standard antibodies. The bias introduced by the synthetic standard is critical because it drastically affects the reading of samples when related to the resulting standard curves. That leads us to conclude that the most accurate measurement of dsRNA in a sample is obtained when the standard used is in line with the sample, meaning that synthetic dsRNA standards will generally introduce a bias when working with IVT samples that are based on natural nucleic acid.

ELISA with natural and synthetic standard

This difference between natural and synthetic dsRNA standards is expected to yield the same bias in any assay using a detection method susceptible of detecting said difference, as shown in the figure below where the same experiment was run with an ELISA.

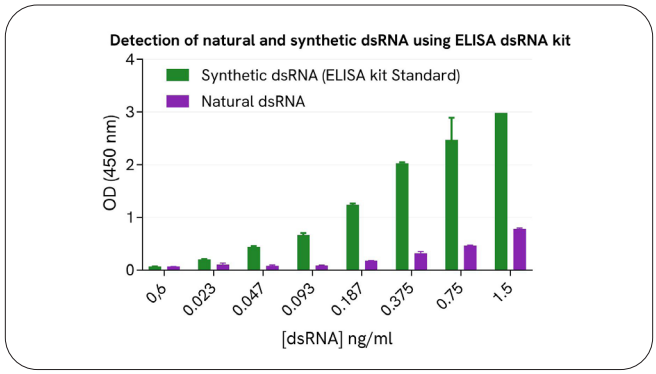

Figure 14: Detection of natural and synthetic dsRNA standards with an ELISA assay.

As expected, the result of the ELISA showed a similar bias introduced by the synthetic dsRNA standard, with a 6-fold overestimation in quantification compared to the natural dsRNA in the assay dynamic range. We also report that in the case of that ELISA, the dynamic range was not well adapted to IVT and called for a high dilution of the samples to be usable.

Working with high ionic strength samples

TE buffers and RNAse-free H_2O are typically used for the *In Vitro* Transcription reaction itself, but the following purification steps can be performed in high-salt buffers (e.g. high levels of KCl), which raises the question of the robustness of dsRNA detection methods in these conditions.

To confront the HTRF dsRNA (IVT) assay in such conditions and mimic actual samples going into purification, the assay was run using the natural dsRNA standard diluted in high ionic strength buffers (TE+/- KCl).

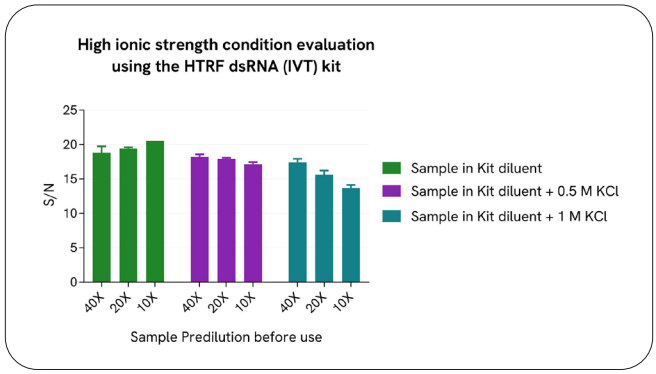

Figure 15: Kit application in high ionic strength IVT condition.

The results show that the dsRNA samples are detected properly and without effect from the buffer for ionic strength up to 0.5M KCl. The assay window in such conditions is similar to that of the dsRNA diluted in TE buffer alone.

For high ionic strength however, the experiment shows that the 10X-dilution results in a lower assay window compared to the TE buffer alone, and that this assay window is improved by increasing the dilution factor. In the case of 1M KCl, the TE buffer assay window is achieved after a 40X dilution.

These results are summarized in the following guidelines, which we recommend following when running the assay.

Table 3: Guidelines to apply dilution factors to IVT samples of high ionic strength.

Ionic strength containing dsRNA sample	Low (0.15M)	High (0.5 M)	Very High (1M)
Guidelines for minimal sample pre-dilution	No minimal predilution required	No minimal predilution required	40X

Specificity and cross-reactivity with other IVT contaminants

Cross reactivities were assessed using other reagents commonly present in mRNA samples during IVT production process, such as single-strand RNA (ssRNA), DNA template (circular and fragments), or T7 polymerase. Titration of each reagent diluted in the kit diluent was performed using concentrations in line with what was found in IVT production processes.

For each reagent and concentration tested, 10 µL of each sample were transferred into a white detection plate (384 low volume), and 5 µL of each HTRF dsRNA (IVT) detection reagents was added. The HTRF signal (Delta Ratio) was recorded after overnight incubation at room temperature (22 °C).

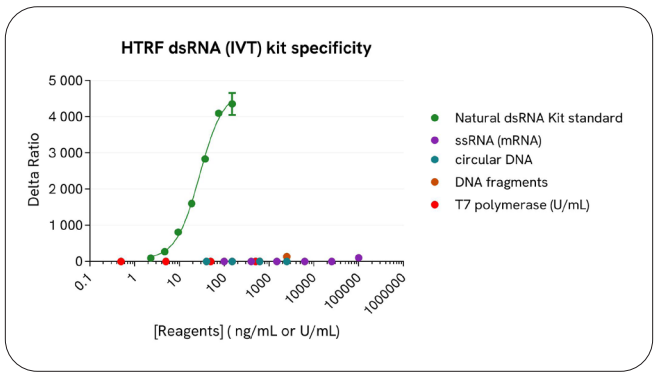

Figure 16: Cross-reactivity of T7 polymerase, ssRNA, plasmidic DNA template, digested DNA template – There was no detection of either at the tested concentrations.

Table 4: Summary of cross-reactivity with other-than-dsRNA components present in IVT products.

Reagents	Range of concentration	Specificity of HTRF dsRNA (IVT) Kit
SSRNA (142 bp same sequence than dsRNA sample)	100 to 100 000 ng/mL	No cross-reactivity
Circular DNA template	40 to 2500 ng/mL	No cross-reactivity
Digested DNA template by DNase I (Fragments)	40 to 2500 ng/mL	No cross-reactivity
T7 polymerase (for enzymatic IVT reaction)	0,5 to 500 U/mL	No cross-reactivity

No detection signal was recorded using for any of the ssRNA, DNA Template, and T7 polymerase samples in comparison with positive controls of dsRNA. In these assay conditions, the kit shows detection specificity for dsRNA over other tested IVT reagents.

Simulation of dsRNA contamination at 0.5% in mRNA IVT mix

Following up on the cross-reactivity experiment, we present here an experiment to demonstrate the lack of interference of any of the previously tested contaminants when we put them together with dsRNA in an IVT sample-like mix. A fixed concentration of a 142 bp natural dsRNA sample (50 ng/mL) was mixed with a 2000-fold more concentrated 142 bp ssRNA sample (10 µg/mL), digested DNA template (10 ng/mL), and 500 U/mL of T7 polymerase. A dsRNA sample at 50 ng/mL was used as reference control.

10 µL of each sample were plated in a 384sv white plate and 10 µL of HTRF (IVT) kit detection reagents mix were added. The HTRF signal (Delta Ratio) was recorded after overnight incubation at room temperature (22 °C).

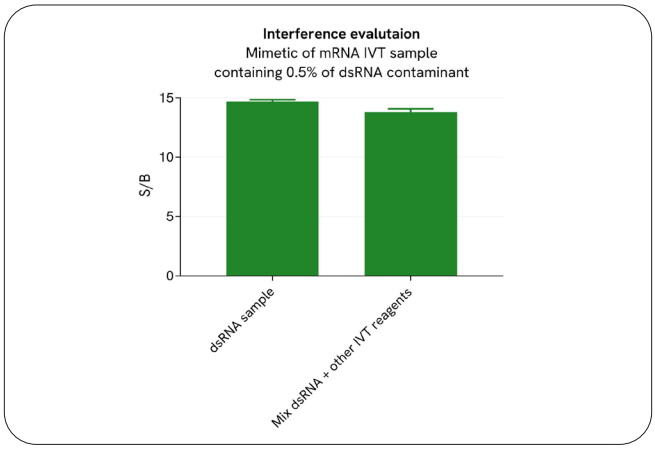

Figure 17: Comparison of dsRNA detection in natural dsRNA sample compared to IVT mix simulation.

Table 5: Summary of IVT mix simulation composition.

Reagent present in the IVT MIX	Concentration
dsRNA (ng/mL)	50
mRNA (ng/mL)	10 000
DNA (ng/mL)	10
T7 polymerase (U/mL)	500

The results show that no significant changes in the HTRF signal were recorded between the natural dsRNA control and the dsRNA + IVT reagent mix, which indicates the absence of interference from common reagents present in IVT samples.