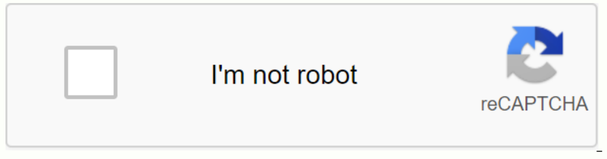

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Qubit cdna concentration

cDNA means complementary DNA, it can be described as double stranded DNA made of RNA. Our ultimate goal is to sequence the RNA we extract - and in our order of choice, we need to turn the RNA back into DNA so that it can be read properly. To this end, we use a technique known as reverse transcription to read and copy RNA content to newly created DNA strands without losing genetic information! The purpose of this experiment was to convert the RNA to cDNA through the inverted yarn priming by oligo-dT. The process consists of three main stages - complementary DNA strand synthesis, RNA digestion and synthesis of the second strand [1]. The steps we carried out are described below: due to the synthesis of complementary DNA strands in addition to the previous polyA to 3'OH, all RNA molecules have a similar sequence at the end of the other 3'end only to the number of added adenine bases. This allows polyT primers (Oxford nanofore) to be dissazeed into RNA templates and thermofishers to initiate transcription. Second, the so-called strand switching primer is added to the reaction. This introduces additional templates to protect the terminal base pairs to compensate for the underrepresentation of 5'ends in the cDNA. RT's extremity deviation activity adds several jailbreak-city bases. The SSP primer is complementary to these bases and acts as an extended template for RT, protecting the terminal base, as well as introducing a sequence of choices to the newly synthesized first strand. RNA template digestive RNA template needs to be removed before synthesizing the second DNA strand. This is done by adding rivonaplis (RNAse cocktail enzyme mix, ThermoFischer) to reactions and incubation. The enzyme mix consists of RNase A and T1. The second strand of synthetic second DNA strands are synthesized using a long amp tak polymerase (NEB). The primer used in the reaction is complemented by the sequence introduced by the SSP and VNP primer. The results were initially achieved, with very low concentrations of cDNA. It was decided to amplify the product using the same primer, which is used for second-strandsynthesis to ensure that cDNA has been synthesized so far. As you can see in Figure 1, you can conclude that the cDNA was actually successfully synthesized after embedding the cDNA as a template amplification PCR product. It has a similar size distribution to the bacteria mRNA, and the strong band at 270 bp was of unclear origin. The rest of the work was to synthesize enough cDNA. Eventually, we were able to achieve a high yield of cDNA after excessive problem shooting, which revealed that the reason for the low yield is inefficient polyadenylation as described here. The amount of cDNA measured by Qubit (Typically, the cDNA amount will be the same as about twice the mass of the input RNA. For 125 ng of mRNA input we synthesized about 300 ng of cDNA (in normal experiments). This amount was sufficient for further experimentation. During this experiment, synthesized RNA-contaminated cDNA was used to prepare a sequencing library. As described here, the quality of input cDNA has begun to be investigated among other factors due to non-optimal sequencing performance. After extensive troubleshooting, it was confirmed that RNA still exists in cDNA samples despite the digestive and cleaning phases. A very large amount of RNA has been found in some samples, often equivalent to input quantities. RNA content was measured using a Qubit HS RNA kit specific to RNA. Unfortunately, an efficient way to remove RNA was not found during the project process, which is the main reason for poor sequencing results. The following sections describe a variety of troubleshooting solutions to investigate RNA contamination. RNA measured in the sample may be made due to the lack of specificity of the Qubit dye. RNA dyes actually have an affinity to DNA and thus show rna in our samples. Experiments: samples containing only RNA or DNA at a concentration of 10 ng / µl was prepared in triple, each sample was measured in two different Qubit kits (RNA HS kit, DNA HS kit, thermo fisher). Results: Measuring RNA (or DNA) with that kit resulted in the same results as expected. About 10 ng and very low amounts of DNA of RNA as shown in Table 1. Similar results were found for DNA samples. When DNA was added to rna, it was the only measurement deflection observed by a decrease in the amount of RNA measured. Table 1. The average of the measured value for each sample. For example, when measuring rna (or DNA) with a corresponding kit, the expected results were generated. About 10 ng and very low amount of DNA of RNA. Sample RNA [ng / µL] DNA [ng / µL] RNA RNA sample 10 ng / µl 9.84 0.64 6.86 - DNA sample 10 ng / µl 0.33 11.3 - 11.7 experiment sDNA and sRNA conclusion only to investigate the interaction between dsDNA and sRNA measurement information (2) is introduced in the measurement information (2). The other question is how RNA: DNA hybrids will be treated by dyes. It is possible to remain in a hybrid form because the RNA is not digested properly. Hybrids could be detected hypothetically by both RNA and DNA-specific kits. Unfortunately, this hypothesis could not be tested because the proven hybrid sample was not available. Having DNA - it was concluded that the RNA mixture does not affect the measurement in an important way. RNA amount is moderately reduced after the addition of DNA to the sample will not explain the presence of RNA in the cDNA sample. What we need to investigate is what rna:DNA hybrids will be like. Measure. So we conclude that RNA measurements in our samples are accurate and that there is rna that exists as ssRNA or RNA:DNA hybrid. RNA is found in the final cDNA product because it is not digested properly. The RNase cocktail includes RNAse A and T1, which require the introduction of nicks into rna. LongAmp Taq polymerase does not degrade these fragments if the tablet beads (AMPure XP, Agencourt) has an affinity for both RNA and DNA, RNA will be found in the sample. Experiments: This problem-solving experiment had three main parts: i) using a longer sleep cure time and / or a larger amount to optimize the protocol for the use of RNase cocktail, and ii) digestion and iii) using RNAse H with a purification bead to determine whether there is an affinity for RNA. 1. A total of eight samples with 125 ng of RNA (in previous cDNA samples that showed RNA contamination, for example there was DNA present in the sample). This experiment is optimized for the time and amount of RNase cocktails. 2. RNA ladder was digested using RNase H, RNA cocktail or both. In addition, each test was prepared in RNase-free water or buffer used during cDNA synthesis. The digested DNA was visualized in the gel. 3. Using 1000 ngAMX beads of the RNA ladder was purified and displayed in the gel. Results: Overall, we saw that subsequent digestion of RNA-contaminated samples succeeded regardless of the concentration used below. Using RNase H and/or RNase cocktails, the digestion of rna ladders shows complete digestion for RNase cocktails. The beads carry over most of the RNA, including rather small fragments (200 bp). The detailed results of each experiment are as follows: Table 1. Table 2. The table shows the treatment for nine samples of RNA and DNA mixtures containing 125 ng of RNA. *This corresponds to the experimental settingused during the actual cDNA synthesis. Sample number incubation time [min] RNase cocktail [µl] RNA treatment after 1* 10 10 undetectable 2 2 10 2 undetectable 3 10 0,5 undetectable 4 30 1 undetectable 5 30 2 undetectable 6 30 0,5 undetectable 7 45 1 detectable 8 45 2 undetectable 9 45 0,5 RNA can be detected. Regardless of treatment, all samples show that all RNA decreases, including the same sample streated as used in the actual experiment. 2. The gel below shows the digestion of the available RNases, RNase H (line 5 and 6) and RNase cocktails (line 7 and 8) or both (lines 8 and 9) and RNA ladders. In this experiment, we can conclude that the buffer does not affect digestion because the samples of water and reaction buffers appear the same. The RNase cocktail is an efficient deterioration of the ladder consisting of ssRNA. RNase H seems The ladder is not digested at all. This is consistent with the described ability of RNase H to digest RNA: DNA hybrids first. Figure 2. Results after digestion with RNases. 3. The gel showed a very important carryover of RNA during purification with AMPure beads (not shown), which corresponds to the manufacturer's information. If the RNA is not digested properly, it is carried with cDNA during the entire library preparation process. Overall, the results of this troubleshooting procedure suggest conflicting results from actual experiments. Here, RNases has been shown to work very efficiently. Most likely, the RNA used to solve the problem has different characteristics in the rna template in the cDNA synthesis (which may be in the form of hybrid or other unusual forms) leading to different digestion results. During cDNA synthesis the discussion was able to achieve a high enough yield, usually more than twice the amount of mRNA entered. This cDNA was used to prepare the sequencing library. As shown later, unfortunately containing an undigested RNA significantly reduced the quality of the sequencing results. Therefore, we spend a significant amount of time finding the source of RNA contamination, as reported in this section. The above experiments show very contradictory results. In section i) after synthesis it can be seen that the treatment of cDNA with RNase cocktail results in a complete interval of RNA in the sample. The RNase cocktail has also been shown to digest rna ladders visualized in gels in Figure 1. The same enzyme is always being used during cDNA synthesis procedures and it remains unclear why it will efficiently digest RNA after synthesis or RNA ladders and will not work during actual synthesis. One hypothesis we considered was the presence of RNA: DNA hybrids, which would reduce the effectiveness of RNase cocktails, which are more efficient for SSDNA digestion. RNase H was therefore added to solve this issue but the permission of the RNA from the sample did not increase significantly. Even more confusingly, digestion was effective in some samples despite the content / treatment is the same. The bottom line was that we successfully managed to synthesize complementary DNA in our mRNA samples, unfortunately it did contain undigested RNA. Protocols must be developed to ensure that all RNA has been removed from the sample before the library can be prepared. Additional troubleshooting should also be performed to determine why digestion is not efficient. Efficient.