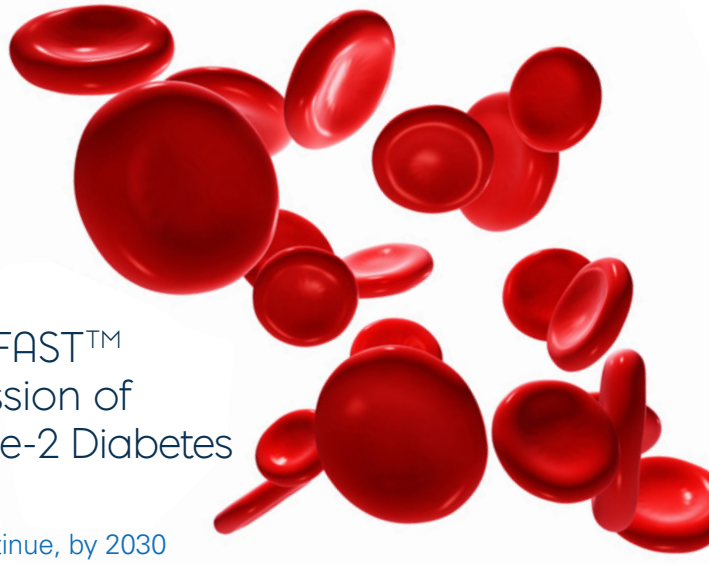
Direct cDNA Synthesis and Amplification from Crude Cell Lysates:

SensiFAST™ cDNA Synthesis Kit and SensiFAST™ Probe Lo-ROX Mix for Studying the Expression of Genetic Markers Related to Obesity and Type-2 Diabetes



Worldwide obesity has nearly tripled since 1975[1] and if trends continue, by 2030 an estimated 38% of the world's adult population will be overweight and another 20% will be obese[2]. The obesity problem is growing and with it, associated chronic diseases such as type-2 diabetes, cardiovascular disease, certain cancers, depression, and disability. Significant research is focused on understanding how to prevent and treat obesity, specifically on the genetic and epigenetic causes. To date, over 60 relatively common genetic markers have been implicated in elevated susceptibility to obesity[3].

SensiFAST™ for cDNA Synthesis and Direct Amplification from Crude Lysate

Gene expression analysis on cultured cells using reverse transcription and quantitative polymerase chain reaction (RT-qPCR) typically involves a long workflow that includes the harvesting of cells, isolation of RNA, removal of co-purified contaminating DNA through DNase treatment, cDNA synthesis, and finally qPCR. The isolation and purification of RNA is a rate-limiting step that poses a significant challenge for high-throughput gene expression analysis.

An approach in which cDNA synthesis is carried out on crude cell lysates instead of on purified RNA samples offers a fast and straightforward alternative. In the following study, SensiFAST™ cDNA Synthesis kit was successfully used to produce cDNA from crude cell lysate and the cDNA templates were used in multiplex and singleplex assays with SensiFAST™ Probe Lo-ROX to examine subtle changes in relative gene expression levels.

By circumventing the need for RNA purification to produce reliable cDNA templates for studies requiring sensitive analysis of gene expression levels, considerable time and cost can be saved and assays can be processed at higher throughput with reduced potential for contamination.

cDNA Synthesis and Direct Amplification from Crude Lysate using SensiFAST™ Probe Lo-ROX Mix

Dietary salt intake is a major determinant of the activation state of the renin-angiotensin-aldosterone system, (RAAS), a key mediator of diabetes-associated atherosclerosis.[4] Angiotensin-converting enzyme 2 (ACE2) is an important modulator of the renin-angiotensin system (RAS) through its role in degrading angiotensin (Ang) II. ACE2 is expressed on the cell surface of several well-known cell lines, including the colon carcinoma cell line Human Caco-2.[5]

The receptor for advanced glycation end products (RAGE) is a type I transmembrane glycoprotein which is implicated in a range of diseases, including Alzheimer's disease, malignancy, diabetic complications, and atherosclerosis. RAGE is basally expressed at high levels in the vascular endothelium and leukocytes, although RAGE expression may be induced in most cell types following injury, inflammation, or hypoxia.[6]

In this experiment (Figure 1) cDNA from crude lysate was prepared from Human Lung cells

Basal Expression Levels of RAGE and ACE2 Detected from cDNA prepared from Crude Lysate and Amplified using SensiFAST™ Probe Lo-ROX Mix

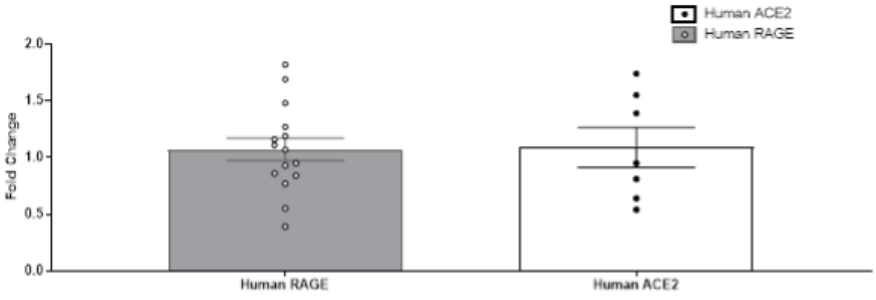


Figure 1. RT-PCR using SensiFAST™ Probe Lo-ROX was used to examine the basal expression level of RAGE in Human Lung cells and ACE2 in Human Caco-2 cells. cDNA was prepared directly from crude lysate, without prior extraction of RNA, and then used in subsequent RT-PCR assays to examine the gene expression levels of RAGE and ACE2. Repeat experiments demonstrate the reliability and repeatability of RT-PCR using SensiFAST™ Probe Lo-ROX from cDNA prepared from crude lysates.

(RAGE) and Human Caco2 cells (ACE2) using previously established methods[6]. Specifically, cells were lysed using 50 µL and 200 µL respectively of pre-prepared lysis buffer[6] and 2 µL of the diluted lysate mixture was added to 4 µL 5 x buffer, 0.2 µL RNase inhibitor, 1 µL MMLV-RT and made up to 20 µL with water. The mix was then incubated at 25 °C for 10 minutes, 42 °C for 60 minutes, 80 °C for 5 minutes and then stored at -20 °C.

To examine the basal expression levels of RAGE in Human Lung cells and ACE2 in Human Caco-2 cells, 1 µL of the cDNA mixture prepared as per above, was added to 1.5 µL of primer mix and 5 µL 2 x SensiFAST™ Probe Lo-ROX Mix and made up to 10 µL with water. PCR was performed using 6 µL of this mix with the following cycling conditions: 95 °C for 20 sec, then 40 cycles of 95°C at 1 sec followed by 60 °C for 20 sec. If the Ct was later than 33 cycles, then 10 µL reaction volumes were used instead. Both RAGE and ACE2 expression levels were consistently detected at similar levels over repeat experiments, demonstrating that this protocol, using SensiFAST™ Probe Lo-ROX Mix for PCR analysis, circumvents the need to purify RNA, allowing cDNA synthesis directly from the cell lysate, saving considerable time, avoiding contamination, eliminating the use of organic solvents, increasing throughput and dramatically reducing costs.

Multiplex Gene Expression Analysis Direct from Crude Lysate using SensiFAST™ cDNA Synthesis Kit and SensiFAST™ Probe Lo-ROX

Several studies have shown that the failure to resolve inflammation may contribute to the progression of many chronic inflammatory disorders. Reducing inflammation through a targeted approach has been suggested as a novel treatment for chronic inflammatory diseases such as diabetes. Lipoxins, a class of endogenously generated mediators, promote the resolution of inflammation and Lipoxin A4 (LXA4) specifically has strong neuroprotective effects in diabetes by reducing the inflammatory reaction in cerebral ischemia/reperfusion injury. It is thought that LXA4 might reduce the levels of monocyte chemoattractant protein-1 (MCP-1) which is often associated with diabetic microvascular or macrovascular complications. In this study, the LXA4 downregulation of MCP-1 expression was examined in mice kidney cells (Figure 2). Specifically, the difference in MCP-1 expression levels after treatment with LX4 or a mimetic (LXM1 or LXM2) was measured using SensiFAST™ Probe Lo-ROX. In this experiment, cDNA from purified RNA was obtained from mouse kidney samples using SensiFAST cDNA Synthesis Kit, where the mice were either in a control or diabetic group, and treated with LXA4, LXM1, or LXM2. Crude lysate was prepared according to the protocol in the SensiFAST™ cDNA Synthesis Kit except the 42°C step was extended to 60 min in the cDNA synthesis reaction in order to obtain a similar yield of cDNA from lysate as from purified RNA.

The relative expression of MCP-1 was measured using SensiFAST Probe Lo-ROX in a multiplex reaction with MCP-1 and 18s as a housekeeping gene. The results show that LXA4 does appear to have a protective effect, reducing the levels of MCP-1, when compared to mimetics.

Effect of Lipoxin A4 (LXA4) on MCP-1 Expression in Mice Kidney Cells

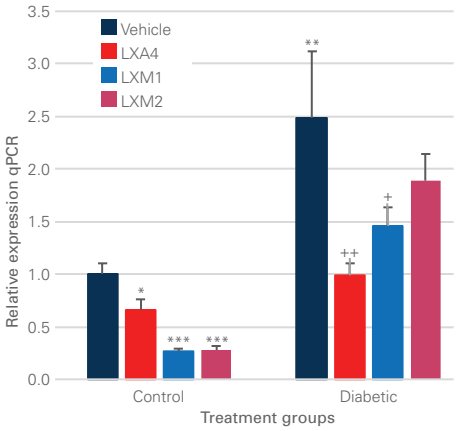


Figure 2. qPCR using SensiFAST™ Probe Lo-ROX to examine the difference in MCP-1 expression levels after treatment with LXA4 or mimetics LXM1 or LXM2. A highly sensitive qPCR mix was required to discriminate the different levels in expression between treatment groups.

Summary

As demonstrated here, SensiFAST™ cDNA Synthesis Kit can be used to generate cDNA directly from crude lysate, without the need to purify the RNA first. This method can save considerable time, increase assay throughput, avoid potential contamination, eliminate the use of organic solvents, and dramatically reduce costs. In addition, SensiFAST™ Lo-ROX Mix can reproducibly amplify gene targets from crudely produced cDNA with high sensitivity in singleplex or multiplex assays. These reagents are ideal for studies requiring amplification from low copy number targets or for deciphering subtle changes in gene expression levels.

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