DireCtQuant

USER GUIDE

DireCtQuant 100D

DNA /RNA /protein solubilisation reagent with Chamelion Carrier™ for

Direct DNA analysis by digital PCR, qPCR, PCR and fluorimetric detection

Direct RNA analysis by RT-digital PCR, RT-qPCR and RT-PCR

Direct protein analysis by Western blot

+ *Chamelion Carrier™*

+ *Stop-the-Time™*

For Research Use Only

Not Intended for any Animal or Human Diagnostic or Therapeutic Use

www.directquant.eu

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ABOUT DIRE*CT*QUANT 100D

The DNA/RNA/protein solubilisation reagent **Dire*Ct*Quant 100D** is conceptually new reagent, which enables quantitative analysis of a biological sample in a very straightforward and precise way. The main advantage of using **Dire*Ct*Quant 100D** is that DNA, RNA and proteins can be analysed from a single sample without the need of prior separation and purification. This reagent provides instant and complete solubilisation of the cells in the sample and permits effective extraction of genomic, plasmid, plastid, mitochondrial and viral DNA as well as messenger, structural (such as ribosomal), transport, microRNA, non-coding and viral RNA and proteins.

The **Dire*Ct*Quant 100D** is developed to address the new challenges of the analysis of nucleic acids by digital PCR. This new technique, based on the partitioning of the PCR in thousands of individual reactions opens completely new possibilities for analysis of very low abundance targets and provides absolute quantification without using standards as a reference. Using digital PCR even samples containing few molecules can provide a meaningful qualitative and quantitative result. This new family of technologies provides completely new challenges for the sample preparation. The possibility to analyse samples containing limited, even single, target molecules new factors should be taken into account. All plastic materials used during the sample manipulations (tubes, pipet tips, *etc.*) have some level of non-specific binding capacity for the nucleic acids in the sample. This adsorption becomes important at low concentrations usually addressed by the digital PCR technologies. This problem is usually solved by inclusion of synthetic carriers or natural nucleic acids carriers.

The non-nucleic acids carriers (synthetic polymers or glycogen) have the disadvantage of not mimicking completely the nature of the nucleic acids and not competing effectively for the binding sites. They also affect the physical properties of the samples and the partitioning process. The natural nucleic acids (such as yeast tRNA or non-homologous DNA) potentially interfere with the subsequent steps of PCR or reverse transcription providing a competitive substrate for the polymerase enzymes. They also require validation per each target analysis as they can always contain a trace amount of homologous targets detectable by the high sensitivity digital PCR technologies. From the other side the natural nucleic acids provide increased fluorescence background in intercalating dye detection chemistries. The digital

PCR present another unique challenge. The quantification is based on effective single molecule distribution of the individual molecules during the partitioning process. This occurs on the majority of the currently available platforms on the market at close to ambient temperature, conditions at which even short, partially homologous regions can bind effectively one to each other. Any target molecule which contains single stranded regions with low homology to other target in the sample will be portioned incorrectly leading to underestimations. This can happen due to non-symmetric dsDNA breaks leaving single stranded regions which can bind other similar regions in degraded DNA samples such as cfDNA, FFPE samples, viral DNA with single stranded regions (adenoviral family), RNA transcripts (especially transcripts from complementary strands in bacterial, plastid, mitochondrial, viral, coding and non-coding RNA´s).

To address these recent challenges **Dire*Ct*Quant 100D** includes Chamelion Carrier™* with the following advantages:

- Nucleic acid carrier- proprietary mixture of synthetic oligonucleotides. This carrier provides effective carrier effect for controlling the non-specific binding to the plastic and non-plastic surfaces the samples come in contact (tubes, pipet tips, and sample homogenization consumables). This mixture controls effectively both double stranded and single stranded nucleic acids binding on competitive basis.
- Non competing carrier-contains non-natural bases, which prevents their usage as substrates during PCR or retro transcription steps.
- The sequence of the carrier provides nearly universal binding capacity for exposed single-stranded regions in both DNA and RNA. This effectively reduces the intermolecular interactions between target molecules leading to quantification errors.
- During the PCR steps the carrier is degraded and do not interfere with the detection of the fluorescence after the PCR step.
- Double release mechanism - due to the unique structure of the carrier. A modification of denaturation step is recommended for the double-stranded DNA detection (see page 5)

*Patent pending

The DNA in **Dire*Ct*Quant 100D** solubilised samples can be directly analysed by digital PCR, quantitative (Real Time) PCR (qPCR), PCR or quantified in solution using **Dire*Ct*Quant DNAQ** (Ref #**DCQDNAQ**). The RNA in the sample can be directly analysed by reverse transcription followed by digital PCR, quantitative PCR (RT-qPCR) or PCR and the proteins can be directly analysed by Western blot.

Note that **Dire*Ct*Quant 100D** is an evolution of our **Dire*Ct*Quant Line** (including Dire*Ct*Quant 100W, Dire*Ct*Quant 100ST and Dire*Ct*Quant 100T-discontinued) and its extraction and cell integrity preservation technologies are not affected. No change in your protocols is expected and no need to revalidate your current protocol developed with **Dire*Ct*Quant Line** is required when switching to **Dire*Ct*Quant 100D** according to our extensive internal testing, except for the PCR thermal protocol (see page 5).

The instant disruption of the structure of the cell and the biopolymers by **Dire*Ct*Quant 100D** effectively blocks the endogenous enzymatic activity of the sample, preventing any degradation of macromolecules and providing exceptional long-term storage conditions for the majority of the samples referred as Stop-The-Time™ technology.

Dire*Ct*Quant 100D provides a one-step, single reagent workflow, reduces the time of analysis and increases the precision and reliability of the study.

PCR thermal protocol modification when using DireCtQuant 100D

Due to the unique double release mechanism a modification of the standard PCR protocol is recommended. This applies for double stranded DNA detection, but not to ssRNA or cDNA detection where is not required. It applies for digital PCR, qPCR and PCR protocols.

An extra annealing-elongation step / denaturation of 2 minutes, should be inserted between the initial denaturation and the PCR cycling protocol.

The extra annealing-elongation step should be as in your optimized protocol. It can consist of single annealing-extension temperature (as in the example below, 2-temperature cycling) or 2 temperatures (as in 3 temperature cycling consisting of annealing temperature and elongation at 72°C)
The extra denaturation step should be 2 minutes at the same temperature as used in your optimized protocol for initial denaturation.

Consult the table below for easy reference:

Example of thermal PCR protocol modification when DireCtQuant 100D is used for dsDNA detection:

	Example standard protocol:	**DireCtQuant 100D** modificación:
Inicial denaturation:	98°C 5min	98°C 5 min
100D dsDNA detection		60°C 10 sec
100D dsDNA detection		98°C 2 min
Denaturation (30-45cycles):	95°C 10 sec	95°C 10 sec
Anneling-elongation (30-45cycles):	60°C 10 sec	60°C 10 sec
Melting curve (qPCR with SYBR Green only)	60 to 95°C 25min	60 to 95°C 25min

TECHNOLOGY BEHIND DIRE*CT*QUANT 100D

Dire*Ct*Quant 100D reagent is stable micro-emulsion at a precise equilibrium state. Upon contact with the biological sample at high temperature the reagent disrupts its structure and instantly induces a long-term suppression of the enzymatic activities. With our innovative **Stop-The-Time™** technology included in this reagent individual biopolymers are encased in a protective shell, which guarantees exceptional long term storage capability. Upon mixing with water an abrupt structural transition is induced, which then permits analysis by enzyme mediated assays such as PCR.

It is important not to alter the composition of the extraction reagent and to use the recommended sample to reagent ratio in order to preserve these qualities.

REAGENTS AND EQUIPMENT TO BE SUPPLIED BY THE USER

- Dounce homogenizer/ Potter-Elvehjem/ bead homogenizer
- Micro centrifuge
- Thermo block capable of maintaining 90°C, preferably with agitation
- Nucleases/proteases-free water
- Reagents required by the following analysis such as PCR, qPCR, RT-PCR, RT-qPCR, Western blot o fluorimetric detection of DNA

REAGENT STORAGE

Dire*Ct*Quant 100D is shipped at ambient temperature. Upon arrival, store at room temperature. Once opened, should be stored at room temperature and used within one month.

Mammalian Tissue Samples Solubilisation

1. **Weigh the tissue and add 3.0 ml of DireCtQuant 100D for every 100 mg of tissue sample.**

   * **NOTE:** *Both fresh and snap frozen in liquid nitrogen tissues can be used. In case of starting from a frozen tissue, proceed directly by dropping the frozen sample in appropriate amount of* ***DireCtQuant 100D****.*

Sample weight	*#DCQ100D*
10 mg	*0.3 ml*
100 mg	*3 ml*
300 mg	*9 ml*
600 mg	*18 ml*

2. **Homogenize completely with Dounce, Potter-Elvehjem or bead homogenizer.**

   ! **CRITICAL:** *Complete homogenization of the tissue is critical. Usually 20 strokes of Dounce homogenizer with tight clearance (approx. 50 µm) are sufficient for soft tissues. For harder tissues use Potter-Elvehjem homogenizer at median speed and avoid creation of bubbles. No particles after homogenization should be visible by eye. Turbidity in case of lipid rich tissues (such as brain, liver, etc.) is expected and normal. If you are trying to solubilise tissues rich in collagen, keratins or cartilage your sample is not going to be completely solubilised and a pellet in step 5. will be observed.* ***DireCtQuant 100D*** *is not intended to break covalent bonds and as a result can´t solubilise that type of extracellular matrix. In spite of this,* ***DireCtQuant 100D*** *will effectively extract the soluble components (DNA, RNA and not cross-linked proteins) into the supernatant if adequate homogenization is achieved. Homogenize as much as possible with the appropriate method using for example bead homogenizer.*

3. **Transfer the lysate to an appropriate vial which can be securely closed, i.e vial with screw cap. Use a vial with a volume similar to the volume of your sample in order to minimise volume changes due to evaporation in the next step.**

4. **Heat for 3 minutes at 90°C with shaking at 750 rpm or manually inverting the vial three to five times during the heat incubation.**

   ! **CAUTION:** *Use appropriate protection when handling hot vials.*

5. **Leave to cool down to room temperature and centrifuge at 5000 rcf for 1 minute.**

6. **If a pellet or lipid layer on the top is observed, transfer the supernatant to a new vial.**

7. **Proceed with the analysis or store at -20°C (see sample storage).**

Mammalian Adherent Culture Cells Solubilisation

1. **Completely remove the cell culture medium by aspiration.**

   ! **IMPORTANT:** *It is not recommended to trypsinise or detach the adherent cells in any other way. This can induce cell loss, RNA degradation or gene expression changes.*

2. **Add a minimum 200 µl of DireCtQuant 100D for every cm^2 of confluent cell layer in a plate.**

   * **NOTE:** *The optimal extraction ratio is 10^6 normal diploid human, rat or mouse cells per 1ml of **DireCtQuant 100D.** The recommended maximum is $2x10^6$ cells/ml of **DireCtQuant 100D.** For some cell types, as polyploid cell lines, the number of cells should be reduced to avoid viscosity.*

3. **Collect completely the cells (using a cell scraper for example) and transfer to an appropriate vial which can be securely closed, i.e vial with screw cap. Use a vial with a volume similar to the volume of your sample in order to minimise volume changes due to evaporation in the following steps.**

4. **Mix the cell lysate with a pipette (pipetting up and down three to five times is usually sufficient) until the sample appears homogeneous.**

   * **NOTE:** *If the lysate is too viscous and is difficult to pipette, this means that the amount of cells is higher than the maximum recommended ratio of $2x10^6$ cells/ml of **DireCtQuant 100D**. In such a case add 50% more **DireCtQuant 100D** to the homogenate and mix again.*

5. **Heat for 3 minutes at 90°C with shaking at 750 rpm or manually inverting the vial three to five times during the heat incubation.**

   ! **CAUTION:** *Use appropriate protection when handling hot tubes.*

6. **Leave to cool down to room temperature.**

7. **Proceed with the analysis or store at -20°C (see sample storage).**

Solubilisation of Mammalian Cells Grown in Suspension Culture

1. **Count the number of cells in the suspension culture.**
   Use haemocytometer or automatic cell counter

2. **Collect the cells by centrifugation at 1000rcf for 1 minute and discard the supernatant completely by aspiration.**

3. **Resuspend the pellet in Dire*Ct*Quant 100D using the optimal ratio of 10^6 diploid human/rat/mouse cells per ml of Dire*Ct*Quant 100D.**

4. **Mix the cell lysate with a pipette (pipetting up and down three to five times is usually sufficient) until the sample appears homogeneous.**

   * **NOTE:** *If the lysate is too viscous and is difficult to pipette, this means that the amount of cells is higher than the maximum recommended ratio of 2x10^6 cells/ml of* ***DireCtQuant 100D****. In such a case add 50% more* ***DireCtQuant 100D*** *to the homogenate and mix again..*

5. **Transfer the lysate to an appropriate vial which can be securely closed, i.e vial with screw cap. Use a vial with a volume similar to the volume of your sample in order to minimise volume changes due to evaporation in the following steps.**

6. **Heat at 90°C for 3 minutes at 90°C with shaking at 750 rpm or manually inverting the vial three to five times during the heat incubation.**

   ! **CAUTION:** *Use appropriate protection when handling hot tubes.*

7. **Leave to cool down to room temperature.**

8. **Proceed with the analysis or store at -20°C (see sample storage).**

Bacterial and Yeast Samples Solubilisation

1. **Count the number of cells in the suspension culture.**
   Use haemocytometer or automatic cell counter.

2. **Collect the cells by centrifugation at 10 000 rcf for 1 minute and discard supernatant completely.**

   * **NOTE:** *Bacteria and yeast cells are covered by a cell wall, this is an extensive cross-linked structure which cannot be solubilised, however the nucleic acids and the soluble proteins will be effectively extracted.*

3. **Resuspend the pellet in DireCtQuant 100D using the optimal ratio of 10^7 bacterial or 10^6 yeast cells per ml of DireCtQuant 100D until appears homogeneous.**

4. **Transfer the lysate to an appropriate vial which can be securely closed, i.e vials with screw cap. Use a vial with a volume similar to the volume of your sample in order to minimise volume changes due to evaporation in the following steps.**

5. **Heat for 3 minutes at 90°C with shaking at 750 rpm or manually inverting the vial three to five times during the heat incubation.**

   ! **CAUTION:** *Use appropriate protection when handling hot tubes.*

6. **Leave to cool down to room temperature.**

7. **Centrifuge at 10 000 rcf for 1 minute and transfer the supernatant to a new vial. Discard the pellet.**

   NOTE: *If the lysate is too viscous and is difficult to pipette, this means that the amount of cells is higher than the maximum recommended ratio of* $2x10^7$ bacterial or $2x10^6$ yeast cells per ml of ***DireCtQuant 100D****. In such a case add 50% more* ***DireCtQuant 100D*** *to the homogenate and return to step 4.*

8. **Proceed with the analysis or store at -20°C (see sample storage).**

Plant Tissue Samples Solubilisation

1. **Weigh the tissue and add 3.0 ml of DireCtQuant 100D for every 100 mg of tissue sample.**

   * **NOTE:** *Plant cells are covered by a cell wall, this is an extensive cross-linked structure which is not intended to be solubilised, however the nucleic acids and the soluble proteins will be effectively extracted when mechanical disruption of the cell wall is achieved.*

Sample weight	***#DCQ100D***
10 mg	*0.3 ml*
100 mg	*3 ml*
300 mg	*9 ml*
600 mg	*18 ml*

2. **Homogenize completely with bead homogenizer.**

   ! **CRITICAL:** *Complete homogenization of the sample material is critical. Optimize the time, size and type of the beads to achieve complete homogenization. Alternatively freeze in liquid nitrogen, grind to powder and add to appropriate amount of* ***DireCtQuant 100D****.*

3. **Transfer the suspension to an appropriate vial which can be securely closed, i.e vials with screw cap. Use a vial with a volume similar to the volume of your in order to minimise volume changes due to evaporation in the following steps.**

4. **Heat for 3 minutes at 90°C with shaking at 750 rpm or manually inverting the vial three to five times during the heat incubation.**

   ! **CAUTION:** *Use appropriate protection when handling hot tubes.*

5. **Leave to cool down to room temperature and centrifuge at 5000 rcf for 1 minute.**

6. **Transfer the supernatant to a fresh vial.**

7. **Proceed with the analysis or store at -20°C (see sample storage).**

Tip: Print this page apart, as a lab bench protocol!

Liquid Samples Processing

Dire*Ct*Quant 100D provide a unique opportunity to analyse the cell-free (circulating, exosome-associated) nucleic acids without the purification steps usually associated with significant nucleic acid loss. With a properly optimized assay, is possible to detect single molecule per µl of sample.

1. **Mix the sample (serum, plasma, cerebrospinal liquid) with equal volume of Dire*Ct*Quant 100D (e.g. mix 1 ml of plasma with 1 ml of Dire*Ct*Quant 100D).**
   For whole blood samples (fresh, freshly frozen or anticoagulated) mix 100 µl of blood with 1 ml of Dire*Ct*Quant 100D.

2. **Use a vial which can be securely closed, i.e vials with screw cap. Use a vial with a volume similar to the volume of your sample in order to minimise volume changes due to evaporation in the next step.**

3. **Heat for 3 minutes at 90°C with shaking at 750 rpm or manually inverting the vial three to five times during the heat incubation.**

   ! **CAUTION:** *Use appropriate protection when handling hot tubes.*

4. **Leave to cool down to room temperature and centrifuge at 5000 rcf for 1 minute. Transfer the supernatant to a fresh vial.**

5. **Proceed with the analysis or store at -20°C (see sample storage).**

SAMPLE STORAGE (Stop-the-Time™ technology)

Biological samples solubilised in **Dire*Ct*Quant 100D** have exceptional storage stability. This provides unprecedented flexibility during analysis procedure. Once extracted, the samples can be even stored at the lab bench if the analysis is completed within a week. This opens also the possibility for performing field extractions, as very simple portable equipment is required for this step, followed by standard ambient temperature transport to the laboratory. This opens a whole new possibility for the environmental research, field diagnostic and food analysis*. Our tests have shown no detectable degradation of **DNA**, **RNA** or **protein** in samples stored in undiluted **Dire*Ct*Quant 100D** under the following conditions:

- at 37°C for a week;
- at room temperature for a week;
- at 4°C for a week;
- at -20°C for 3 months;
- at -80°C for at least 6 months;
- up to eight heat (90°C) and cool (RT) cycles;
- up to eight freeze(-20°C)and thaw (RT)cycles.

The DNA degradation was assessed by qPCR, the RNA degradation was assessed by RT-qPCR and protein degradation was assessed by densitometry comparison of protein bands separated by gel electrophoresis. We estimate that the precision of the assays used is better than 10%

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SAMPLE ANALYSIS

These guidelines are provided as a starting point for your consecutive analysis. They will require the corresponding reagents and equipment, which are not supplied with **Dire*Ct*Quant 100D**.

DNA analysis

– DNA quantity measurement

DNA quantity in samples prepared with **Dire*Ct*Quant 100D** can be measured using fluorescent DNA stain **Dire*Ct*Quant DNAQ** (Ref **#DCQDNAQ**). As the DNA amount per cell is a constant (with few exceptions) DNA quantification allows precise estimation of the total number of cells per µl of sample solubilised with **Dire*Ct*Quant 100D**. The calculated number of cells per volume of sample is important in order to use the optimal cells/volume ratio in the PCR/qPCR/RT-qPCR and Western blot analysis.

For more information see **Dire*Ct*Quant DNAQ** protocol

– Digital PCR/qPCR/PCR

(Please consult page 5. for modification of the PCR thermal protocol)

Samples prepared with **Dire*Ct*Quant 100D** can be directly analysed by digital PCR/qPCR or PCR.

For 20 µl reaction:

1. **Add up to 1 µl of the sample solubilised with Dire*Ct*Quant 100D.**
2. **Add Forward and Reverse primers or hydrolysis probe depending of the chemistry of your assay.**
3. **Add PCR grade water**
4. **Add digital PCR mix /qPCR mix/PCR mix.**
5. **Add thermo stable polymerase if not included in the digital PCR/ qPCR/PCR mix.**

! **IMPORTANT:** *It is very important to follow the exact order of adding reagents to the reaction. Polymerase enzyme/ Polymerase enzyme containing mix should be the **last** component to add as undiluted **Dire*Ct*Quant 100D** reagent will inhibit the polymerase activity. For digital PCR, if required restriction enzyme digestion can be performed directly in the reaction mixture, following the producer protocol.*

! **IMPORTANT:** *The volume of the **Dire*Ct*Quant 100D** reagent should not exceed **1/20** of the final volume of the reaction.*

NOTE: *For steps 2 to 5 check the instructions provided by your digital PCR/qPCR/PCR kit and equipment manufacturer.*

* **NOTE:** *Suggested format for digital PCR/qPCR or PCR analysis is **1000 cells per reaction** (for example 1µl **DireCtQuant 100D** sample extracted at optimal conditions). This guarantees low variability and a confident detection of expression as low as single gene/transcript copy per cell. Using significantly higher number of cells per reaction does not give any advantage as this is likely to inhibit the PCR reaction. For more information about how to calculate number of cells per volume of sample please consult **DireCtQuant DNAQ** protocol.*

- Ultrafast sample preparation for genotyping purposes **(Please consult page 5. for modification of the PCR thermal protocol)**

This minimalistic procedure is designed to prepare genotyping ready sample from minimal amount of tissue in less than 5 minutes. It will generate sample sufficient for at least fifty genotyping reactions from a single sample.

1. **Use a small piece of tissue – mouse/rat tail clip of not more than 1.5 mm, a single ear punch or similar. Take care to clean well the instruments used for sample collection to avoid cross-contaminations.**
2. **Insert the sample into 0.2 ml or 0.5 ml tube containing 55 µl DireCtQuant 100D. Make sure the tissue sample is completely submerged in the reagent. Spin down the tissue sample if required.**
3. **Heat for 3 minutes at 90°C with shaking at 750 rpm or manually inverting the vial three to five times during the heat incubation.**

! **CAUTION:** *Use appropriate protection when handling hot tubes.*

4. **Leave to cool down to room temperature and centrifuge at 5000 rcf for 1 minute.**
5. **Transfer 50 µl of the supernatant to a fresh vial.**
6. **Proceed with the analysis or store at -20°C (see sample storage).**
7. **Use 1 µl per 20 µl PCR reaction.**

For very high resolution electrophoresis use our system for nucleic acids electrophoresis (Ref #DCQNAE). For PCR products of less than 100 bp, usually employed for genotyping purposes, resolution of single base pair differences in 10 min. is achievable.

RNA analysis

Samples prepared with **DireCtQuant 100D** can be directly analysed by RT-digital PCR / RT-qPCR or RT -PCR

! **IMPORTANT:** *It is very important to use **exon-exon spanning** primers or probes when analysing RNA in samples prepared with **DireCtQuant 100D**, as no separation of DNA and RNA is performed and they are both present in the sample.*

We strongly recommend the use of one-step RT-digitalPCR / RT-qPCR kits over two-step RT-digital PCR / RT-qPCR kits.

– One step RT-digital PCR / RT-qPCR

For 20 µl reaction:

1. **Add up to 1 µl of the sample solubilised with DireCtQuant 100D.**
2. **Add Forward and Reverse primers or hydrolysis probe.**
3. **Add PCR grade water**
4. **Add RT-digital PCR / RT-qPCR mix**
5. **Add RT polymerase**

! **IMPORTANT:** *It is very important to follow the exact order of adding reagents to the reaction. Polymerase enzyme/ Polymerase enzyme containing master mix should be the **last** component to add as undiluted **DireCtQuant 100D** reagent will inhibit the polymerase activity.*

! **IMPORTANT:** *The volume of the **DireCtQuant 100D** reagent should not exceed **1/20** of the final volume of the reaction.*

* **NOTE:** *For steps 2 to 5 check the instructions provided by your PCR/qPCR kit and equipment manufacturer.*

* **NOTE:** *Suggested format for PCR/qPCR analysis is **1000 cells per reaction** (for example 1000 cells per 1 µl **DireCtQuant 100D**). This guarantees low variability and a confident detection of expression as low as single gene copy per cell. Using significantly higher number of cells per reaction does not give any advantage as this is likely to inhibit the PCR reaction. For more information about how to calculate number of cell per volume of sample please consult **DireCtQuant DNAQ** protocol.*

– Two step RT-qPCR

For 20 µl reactions:

- First step (Reverse transcription):

1. **Add up to 1 µl of the sample solubilised with DireCtQuant 100D.**
2. **Add gene specific primer, random hexamers or oligo dT primers if not included in the mastermix.**
3. **Add PCR grade water.**
4. **Add RT buffer/ master mix.**
5. **Add RT polymerase.**

! **IMPORTANT:** *It is very important to follow the exact order of adding reagents to the reaction. Polymerase enzyme/ Polymerase enzyme containing master mix should be the **last** component to add as undiluted **DireCtQuant 100D** reagent will inhibit the polymerase activity.*

! **IMPORTANT:** *The volume of the **DireCtQuant 100D** reagent should not exceed **1/20** of the final volume of the reaction.*

* **NOTE:** *For steps 2 to 5 check the instructions provided by your PCR/qPCR kit manufacturer.*

* **NOTE:** *Suggested format for PCR/qPCR analysis is **1000 cells per reaction** (for example 1µl **DireCtQuant 100D** sample extracted at optimal conditions). This guarantees low variability and a confident detection of expression as low as single transcript copy per cell. Using significantly higher number of cells per reaction does not give any advantage as this is likely to inhibit the PCR reaction. For more information about how to calculate number of cell per volume of sample please consult **DireCtQuant DNAQ** protocol.*

* **NOTE:** We suggest the usage of gene specific primer strategy, as we have observed that this will provide the best sensitivity.

- Second step (digital PCR / qPCR):

For 20 µl reactions:

1. **Add up to 2 µl of the product (cDNA) of the first reaction.**
2. **Add Forward and Reverse primers or hydrolysis probe.**
3. **Add PCR grade water**
4. **Add digital PCR /qPCR buffer mix**

Add thermo stable polymerase if not included in the buffer as PCR/qPCRin the mix.

Protein analysis

- **SDS-PAGE**

We strongly recommend the use of **Dire*Ct*Quant ELM** (Ref **#DCQELM**) loading buffer for preparing the samples extracted with **Dire*Ct*Quant 100D** for SDS-PAGE.

For total volume of 40 µl:

1. **Prepare the sample by mixing:**

   - **Add up to 31 µl of the sample prepared with Dire*Ct*Quant 100D**
   - **Add 8 µl of 5x loading buffer (Dire*Ct*Quant ELM)**
   - **Add 1 µl of a reducing agent of your choice (2-mercaptoethanol, 1M dithiothreitol or 0.5M TCEP).**

   * **NOTE:** ***2-Mercaptoethanol/β-mercaptoethanol (2-ME)*** *is usually supplied as a 14M solution (pure liquid) which will result in 360 mM final concentration if 1 µl is used for the sample preparation.* ***Dithiothreitol (DTT)*** *should be freshly prepared as a 1M solution in water (the resulting final concentration will be 25 mM).* ***Tris(2-carboxyethyl)phosphine) (TCEP)*** *should be prepared as 0,5M solution at neutral pH . The stock is stable at room temperature (the resulting final concentration will be 12.5mM). We recommend the use of TCEP, as it is without-odour, non volatile and irreversible reducing agent.*

   * **NOTE:** *Suggested format for Western blot is 10,000 - 30,000 cells per 5 mm wide lane, depending on the detection condition. This corresponds to 10-30 µl* ***Dire*Ct*Quant 100D*** *sample extracted at optimal conditions.*

   ! **IMPORTANT:** *Please use only non-diluted* ***Dire*Ct*Quant 100D*** *to equalize sample volumes. Don´t use water, this will interfere with the migration of proteins and tracking dye characteristics.*

   ! **IMPORTANT:** *The* ***Dire*Ct*Quant ELM*** *should be used ONLY with samples extracted with* ***Dire*Ct*Quant 100ST, Dire*Ct*Quant 100W*** *or* ***Dire*Ct*Quant 100D*** *buffers.*

2. **Heat for 3 minutes at 90°C with shaking at 750 rpm.**

3. **Let the sample to cool down to room temperature and load on the gel.**

   * **NOTE:** *The* ***Dire*Ct*Quant ELM*** *loading buffer contains a temperature indicator which serves as tracking dye as well. The colour of the sample is changing as follows:*

   – ***ORANGE*** *- during the incubation at 90°C indicating proper reduction/protein denaturalization conditions.*

– ***RED*** *- when room temperature is reached and sample is ready for loading and during the electrophoresis.*
– ***PINK*** *- when stored at -20°C.*

Some lots of reducing reagents contain impurities which can interfere with the sensitivity of the temperature indicator, visible by the change of the colour of the sample at room temperature after adding the reducing agent to the sample. In this case you will not observe the clear colour transitions but if proper temperature incubation is performed the sample preparation will be successful.

* **NOTE:** *The tracking dye front is **RED** and clearly defined compared with traditional bromphenol blue-containing tracking dyes. The dye migrates in front of the leading ion in the Laemmli system. No residual binding to proteins is observed as in some cases with bromphenol blue. The absorption spectrum is specifically selected to not interfere with in-gel or membrane fluorescence detection by infrared scanners. The loading media is completely compatible with Laemmli system and MOPS/MES neutral electrophoresis systems. In case of BisTris-gels used in combination with MES buffer, the dye front is not as sharp and the colour can be yellow, but the electrophoresis assay will be completed correctly in spite of this.*

DIRE*CT*QUANT SOLUBILIZATION REAGENT COMPATIBILITY

Dire*Ct*Quant 100D solubilization reagent is found to be fully compatible with a broad spectrum of kits available on the market for applications, including PCR, qPCR and RT-qPCR

PCR	*qPCR*	*RT-qPCR*	*Digital PCR*
DNA AmpliGel (BioTools)	**SsoAdvanced Universal SYBR green/probes Supermix** (Bio-Rad)	**TaqMan® RNA-to-Ct™ 1-Step Kit** (Applied Biosystems)	**QX family of digital droplet PCR (ddPCR) instruments and compatible reagents** (Bio-Rad) (extensively tested on QX200)
DNA AmpliTools (BioTools)	**iTaq Universal SYBR Green/probes supermix** (Bio-Rad)	**iTaq Universal One-Step Kit** (Bio-Rad)	**QIAcuity Digital PCR instruments and compatible reagents** (Qiagen) (extensively tested on QIAcuity One, 2plex)
AmpliToolsHotSplit (BioTools)	**SsoFast Evagreen Universal Supermix** (Bio-Rad)	**iScript One-Step** (Bio-Rad)	
iTaq (Bio-Rad)	**PerfeCTa line** (QuantaBio)		
AccuStart (QuantaBio)	**qLUMEN™ MasterMix** (GQUENCE)		

GENERAL STATEMENT

Handle our products in accordance with safe laboratory practices: **Wear suitable protective gloves, eyewear and clothing.**

Please read and understand the MSDS data provided and updated at **www.directquant.eu**

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In case of any doubt please feel free to contact DireCtQuant team at: **customersupport@directquant.eu**