

User Manual



TrulyStem kit

for Human Embryonic Stem Cell Characterization

Version 1.2 — March 2010

For use in quantitative real-time PCR



tatabiocenter

Table of contents

Background	4
Contents	5
Additionally required materials and devices	6
Storage	6
Amplification protocol	6
Pipetting protocol	7
Quantification of expression index	7
Calculation example	8
Troubleshooting	9
References	10
License information	10
Contact	10
Other products from TATAA	11

Background

Human embryonic stem cells (hESC) show, besides their importance in basic research, promising future applications including cell replacement therapies, drug discovery and toxicology testing.

The most important characteristic of ESCs is their pluripotency, or ability to differentiate into all three primitive cell lineages in the body -- endoderm, ectoderm and mesoderm. In other words, they can develop into each of the more than 200 cell types of the adult body when given sufficient and necessary stimulation for a specific cell type. Researchers working with hESCs must continually screen them to ascertain pluripotency.

Populations of pluripotent hESC can be derived from the inner cell mass (ICM) of blastocysts or earlier morula stage embryos and have the capacity for indefinite, undifferentiated proliferation in vitro. Differentiation of hESC may occur spontaneously in vitro, especially during sub-optimal culture conditions. In addition, hESC can be hoaxed to differentiate in a directed fashion along specific pathways forming a variety of specialized cell types. However, relatively little is currently known about how to control and manipulate hESC differentiation to produce exclusive populations of specific cell types.

House keeping genes (HKGs) are assumed to be constitutively expressed at a constant level and are therefore frequently used for normalization of gene expression data. Synnergren, J. et al (2007) (13) have showed that commonly used HKGs that have been used in studies of somatic cells are not stably expressed in hESC. They defined a novel set of HKGs specifically for hESCs.

Human ESC are most often grown on a feeder layer of embryonic fibroblasts and require the presence of basic Fibroblast Growth Factor. Without optimal culture conditions or genetic manipulation, embryonic stem cells will rapidly differentiate. A human embryonic stem cell is defined by the presence of several transcription factors and cell surface proteins. The transcription factors Oct-4, Nanog, and Sox2 among others are commonly used to identify hES cells. The cell surface antigens most commonly used to identify hES cells are the glycolipids SSEA3 and SSEA4 and the keratan sulfate antigens Tra-1-60 and Tra-1-81. There is a general lack of rapid, sensitive and quantitative methods for the detection of differentiating human embryonic stem cells.

The TrulyStem kit is a robust and sensitive multi-marker quantitative real-time PCR method, with which you can measure 13 genes regulated upon differentiation. The relative differentiation state of the cells can be determined by calculating a collective expression index based on the mRNA levels of the genes.

Contents

- Control cDNA from differentiated and non-differentiated stem cells, cell lineage AS002. 13 rxns, one reaction for each gene.
- Primer solutions for 13 stem cell differentiation markers. 100 rxns.

Gene	Full Name	Marker for	Expected regulation upon differentiation
Oct-4	POU domain, class 5, transcription factor 1	pluripotency state	down (3)
Nanog	Homeobox transcription factor Nanog	pluripotency state	down (4)
Sox-2*	Transcription factor SOX-2	pluripotency state	down (5)
Cripto	Teratocarcinoma-derived growth factor 1	mesoderm lineage	down (8)
Gdf-3	Growth/differentiation factor 3	pluripotency state	down (6)
βIII Tub	Tubulin beta-3 chain	ectoderm lineage	up (11)
AFP	Alpha-fetoprotein	endoderm lineage	up (7)
NESTIN	NESTIN	ectoderm lineage	up (12)
DESMIN	DESMIN	mesoderm lineage	up (10)
OC90	Otoconin 90	undifferentiated state	down (9)
Lin28	Zinc finger CCHC domain-containing protein 1	undifferentiated state	down (9)
DNMT3B	DNA (cytosine-5)-methyltransferase 3B	undifferentiated state	down (6)
hTERT	human telomerase reverse transcriptase	undifferentiated state	down (6)

*The SOX-2 gene has only one exon. Consequently the assay may amplify genomic DNA.

Primers were designed to form minimum amount of primer-dimer and to span exon-boundaries not to amplify genomic DNA. Efficiency of amplification was determined for each assay on VisiBlue Master Mix containing SYBR Green I. Previous results with OC90 have shown that depending on when the differentiated stem cells (AS002) are harvested the transcription might be down or up regulated. For the cell lineage SA002, DESMIN exhibits slight down regulation or no difference upon differentiation. Literature indicates up regulation for DESMIN but can vary with cell lineage.

Additionally required materials and devices

- Real-time PCR instrumentation.

This kit has been validated on several different instrument platforms. It is suitable for use on all qPCR instruments.

- Mastermix or mastermix components

This kit has been validated using VisiBlue MasterMix. The kit should also be compatible with most other commercially available mastermixes.

- Pipettes and tips
- Vortex and centrifuge
- Sample cDNA

It is recommended to use cDNA of high quality. Quality of RNA can be measured prior to cDNA synthesis using Agilent 2100 Bioanalyzer or BioRad Experion.

Storage

The primer solutions can be stored at +4°C for a period of 12 months. For long term storage -20°C is recommended. The mastermix can be stored at -20°C for six months and at +4°C for shorter periods. Repeated freeze-thaw cycles are not recommended.

Amplification protocol

The amplification protocol below is recommended together with 10 min at 95°C for enzyme activation, if VisiBlue MasterMix is used.

After amplification it is recommended to perform dissociation curve analysis from 65°C to 95°C. This is programmed according to the instrument manufacturers' instructions.

UNG step (optional)	according to mix instructions		
Enzyme activation	10 min, 95°C or according to mix instructions		
Cycling (x40)	denaturation	20s	95°C
	annealing	20s	60°C
	elongation	20s	72°C

Pipetting protocol

We recommend that each sample is quantified in duplicate or triplicate for each gene in the kit. The different genes will be down or up regulated more or less, due to differentiation, depending on stem cell lineage. Measurements of all genes give a complex analysis of the differentiation state. Often it may be sufficient to measure a subset of genes to get a good estimation of the differentiation state. A suggestion is to evaluate this for each cell line. For the control cDNA run a reaction with each gene and calculate the expression index. Save the Ct values and calculations for comparison with your samples.

Make a mastermix for each gene according to the protocol below. Make at least 1 rxn extra so that you do not run out of mastermix during the pipetting. Recommended amount of cDNA is 10ng/reaction. Larger volumes of cDNA may be added. In this case change the volume of water accordingly. Use 2 µl of the control cDNA included in the kit, which corresponds to 10ng. It is recommended to use the same amount of cDNA for all samples.

Component	1 rxn
PCR-Grade water	7,2µl
Primermix	0,8µl
Mastermix (2x)	10µl
cDNA	2µl
Final Volume	20µl

Quantification of gene expression

Quantification of gene expression is based on the Ct value for each sample. The Ct values are calculated as the average of duplicate or triplicate measurements. A mathematical model, previously described in detail (1), can be used to determine the expression ratio of two or more genes. The general mathematical formula to calculate an expression index, based on the geometric average of several reporter genes, is given by (equation 1)(2):

$$\text{Index} = K_{RS} \frac{\sqrt[n]{(1+E_{gene(1)})^{Ct_{gene(1)}} \times (1+E_{gene(2)})^{Ct_{gene(2)}} \times \dots \times (1+E_{gene(n)})^{Ct_{gene(n)}}}}{\sqrt[m-n]{(1+E_{gene(n+1)})^{Ct_{gene(n+1)}} \times (1+E_{gene(n+2)})^{Ct_{gene(n+2)}} \times \dots \times (1+E_{gene(m)})^{Ct_{gene(m)}}}}$$

E is the PCR efficiency, Ct is the threshold cycle, and n and (m-n) are the numbers of genes that are up and down regulated, respectively, upon differentiation of hESCs. The PCR efficiencies were evaluated from dilution series of purified PCR products [1]. All the primers in the kit have an efficiency of about 90 %.

KRS is the relative sensitivity constant that, among other things, accounts for the differences in the fragment lengths of templates. It does not affect relative comparisons of samples and is therefore not necessary to determine.

Calculation example

A qPCR experiment with SSEA+ (differentiated) and SSEA- (non-differentiated) human embryonic stem cells were performed to determine the differentiation state. The cells (SA121) were cultured and harvested on day 7 and fractionated using SSEA-4-antibody-coated magnetic beads, followed by RNA extraction and cDNA synthesis. The relative RNA levels of Oct4, Nanog, Cripto and AFP were determined using QPCR. The genes were run in duplicates, and the expression index was calculated with the data in the table.

Gene	Template	Ct	Average
AFP	SSEA+	24,5	24,585
		24,67	
	SSEA-	21,8	21,73
Oct4	SSEA+	13,17	13,1
		13,03	
	SSEA-	14,25	14,12
Nanog	SSEA+	16,82	16,775
		16,73	
	SSEA-	18,19	18,02
Cripto	SSEA+	14,74	15,28
		15,82	
	SSEA-	16,84	17,1
		17,36	

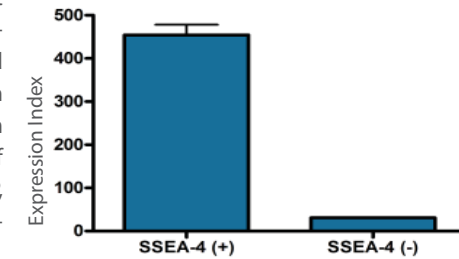
An average value for each sample was calculated. By using equation 1 and the average values together with 90% efficiency two expression indexes were calculated, one for SSEA+ and one for SSEA-. All the primers in the kit have approximately 90% efficiency.

$$\text{Index}_{\text{SSEA4+}} = \frac{(1+0,9)^{24,585}}{\sqrt[3]{(1+0,9)^{13,1} \times (1+0,9)^{16,775} \times (1+0,9)^{15,28}}} = 454,4133$$

$$\text{Index}_{\text{SSEA4-}} = \frac{(1+0,9)^{21,73}}{\sqrt[3]{(1+0,9)^{14,12} \times (1+0,9)^{18,02} \times (1+0,9)^{17,1}}} = 30,3417$$

The equations show how to calculate expression indexes. The mathematical formula used is equation 1.

The graph shows the great difference in expression index comparing SSEA+ and SSEA- cells. The expression index was calculated from the relative RNA levels of Oct4, Nanog, Cripto and AFP, which were determined using QPCR.



Troubleshooting

• I do not get any amplification/signal?

The instrument may not have been programmed correctly or there may be a problem with your mastermix. Evaluate if the problem is in the detection or the amplification by running the samples on gel. Run a new test using the positive control DNA provided with the kit.

• My negative controls give a positive amplification?

Since the assays are based on SYBR Green detection, all dsDNA is detected, including primer-dimers. Depending on which mix is used, varying amounts of primer-dimers are formed. Typically negative controls give Ct-values well above 35 cycles. Evaluate whether amplification is due to primer-dimers or contamination using melt-curve analysis.

• My samples have same/higher Ct-value than my negative controls?

This indicates that you have added too little cDNA. Add more cDNA and try again. The cDNA may be of low quality. Check the quality of the RNA before doing cDNA synthesis.

• My replicates are not very tight?

With good quality cDNA and good pipetting technique, very high reproducibility is possible. Low amounts of cDNA can lead to higher variation. Also, low quality cDNA can lead to big differences between replicates. Check the accuracy and reproducibility of your pipettes.

• I get a positive amplification from genomic DNA

Where possible the assays in the TrulyStem kit have been designed to span exon-boundaries. However, when intron-less pseudogenes are present in the genome, genomic DNA may still give a positive amplification. Try removing DNA contamination by DNase treatment of the RNA sample.

References

1. Stahlberg A, Aman P, Ridell B et al. Quantitative real-time PCR method for detection of B-lymphocyte monoclonality by comparison of kappa and lambda immunoglobulin light chain expression. *Clin Chem* 2003;49:51–59.
2. Noaksson K. et al (2005) Monitoring differentiation of human embryonic stem cells using real-time PCR, *Stem Cells* 23(10) 1460-1467
3. Nichols, J. et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor OCT4. *Cell* 95, 379–391 (1998).
4. Chambers, I. et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113, 643–655 (2003).
5. Avilion, A.A. et al. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* 17, 126–140 (2003).
6. Sperger, J.M. et al. Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proc. Natl. Acad. Sci. USA* 100, 13350–13355 (2003).
7. Dziadek, M.A. & Andrews, G.K. Tissue specificity of alpha-fetoprotein messenger RNA expression during mouse embryogenesis. *EMBO J.* 2, 549–554 (1983).
8. Bhattacharya, B. et al. Gene expression in human embryonic stem cell lines: unique molecular signature. *Blood*, 15 April 2004, Vol. 103, No. 8, pp. 2956-2964.
9. Richards, M. et al. The Transcriptome Profile of Human Embryonic Stem Cells as Defined by SAGE. *Stem Cells* 2004;22:51-64
10. Li, H, et al. Regulation of the mouse desmin gene: transactivation by MyoD, myogenin, MRF4 and Myf5 *Nucleic Acids Research*, 1993, Vol. 21, No. 2 335-343
11. Toumadje, A. et al. Pluripotent Differentiation In Vitro of Murine ES-D3 Embryonic Stem Cells. *In Vitro Cell. Dev. Biol.--Animal* 39:449M,53, November=December 2003
12. Uchida, N. et al. Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci U S A.* 2000 Dec 19;97(26):14720-5
13. Synnergren, J. et al. Differentiating Human Embryonic Stem Cells Express a Unique Housekeeping Gene Signature. *Stem Cells* Vol. 25 No. 2 February 2007, pp. 473 -480

License information

PCR is covered by several patents owned by Hoffman-La Roche Inc. and Hoffman-LaRoche, Ltd. Purchase of the TrulyStem kit does not include or provide a license with respect to any PCR-related patent owned by Hoffman-La Roche or others. TATAA Biocenter does not encourage or support the unauthorised or unlicensed use of the PCR process.

Contact

For more information about the products please contact us at info@tataa.com or visit our homepage, www.tataa.com.

Other products from TATAA

Human Endogenous Control Gene Panel

A panel containing primer sets for 12 commonly used human reference genes. A perfect product for finding the most optimal reference gene for your samples. GenEx Standard software is also included in the kit.

Mouse Endogenous Control Gene Panel

A panel containing primer sets for 12 commonly used mouse reference genes. A perfect product for finding the most optimal reference gene for your samples. GenEx Standard software is also included in the kit.

Chromofy A new dye for qPCR and HRM

Chromofy is a dye developed by TATAA Biocenter which can be used as an un-specific dye in qPCR applications and High-Resolution Melt. The dye shows a strong fluorescence increase when bound to dsDNA and has similar excitation and detection wavelengths as SYBR and FAM.

GenEx software

A software for gene expression analysis. GenEx provides the appropriate tools to analyze real-time PCR gene expression data and to extract valuable information from the measurements.

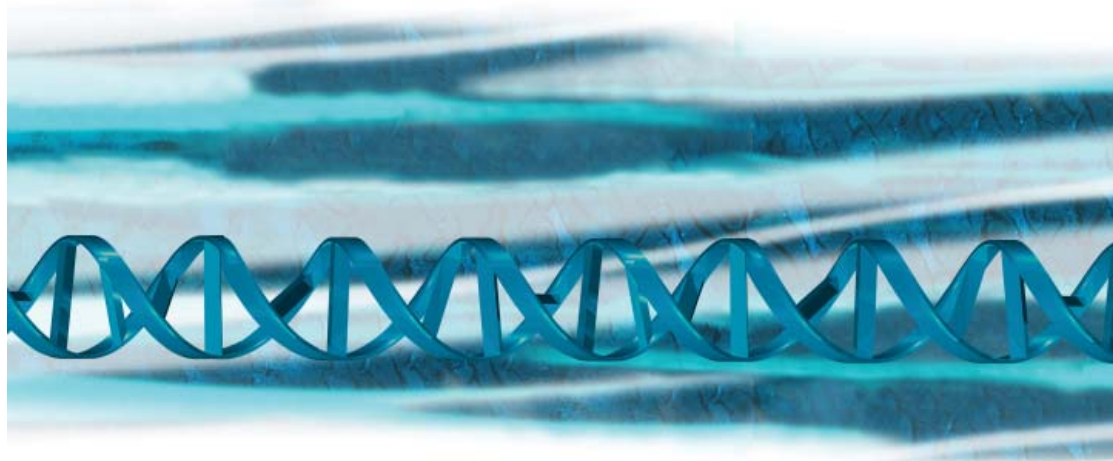
CelluLyser - for rapid and easy lysis and cDNA synthesis

The CelluLyser Lysis and cDNA Synthesis Kit enables you to generate cDNA from small samples with minimal losses and hands-on time, allowing even a single cell to be analysed. A rapid and sensitive lysis is followed by reverse transcription without the need of purification.

VisiBlue mastermix colorant

The VisiBlue mastermix colorant enables you to quickly color your favourite qPCR mastermix to easily visualize where the reagent has been added to your plates and tubes. VisiBlue is very easy to use by a simple addition to your mastermix.

Express your genius



TATAA Biocenter, with offices in Gothenburg, San Francisco and Prague, is the leading provider of real-time PCR services and the prime organizer of real-time PCR workshops globally. TATAA Biocenter conducts commissioned research and training within field of molecu-

lar diagnostics and gene expression analysis, along with developing real-time PCR expression panels. TATAA Biocenter has great experience and expertise in high resolution gene expression profiling, pathogen detection, and small sample/single cell analysis.



TATAA Biocenter AB
Odinsgatan 28, 411 03 Göteborg
Tel: +46 31 761 57 00, Fax: +46 31 152890
E-mail: info@tataa.com, Website: www.tataa.com