

Asterand's Gene Expression Profiling Methodology

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METHODS

1 Tissue

Asterand uses human tissues for research activities within its laboratories. These are donated in an ethical manner through partnership with medical intermediaries, hospitals and tissue banks. The transcription profiles are generated using total RNA samples isolated from tissues deemed to be non-diseased. All tissues undergo clinico-pathological assessment by a professionally qualified pathologist.

2 Quality control (QC)

Asterand follows good scientific practice by working according to Standard Operating Procedures, by ensuring that members of staff are appropriately trained, and by ensuring that all equipment undergoes routine servicing and calibration in accordance with the manufacturers' recommendations.

No claim of compliance to the standards of Good Laboratory Practice is made.

3 Gene Expression Profiling at Asterand

Asterand's major gene expression profiling offering is XpressWay™ Profiles. These Profiles consist of quantitative measurement of the expression of target genes in each of 72 different human non-diseased tissue types. The tissues have been chosen to represent the major organ systems of the human body, and each tissue is sourced from three different donors. The collection of tissues used on an XpressWay™ Profile is detailed in Appendix I.

In addition to XpressWay™ Profiles, Asterand undertakes customized gene expression profiling studies that encompass key therapeutic areas and may comprise profiling in both diseased and non-diseased human tissues.

3.1 RNA Isolation and Quality Control (QC)

Total RNA is isolated from the tissues using standard methodologies according to the suppliers' protocol, or with in-house adaptations. QC criteria that must be met for RNA to be suitable for use in cDNA synthesis for Asterand's quantitative gene mapping studies are:

- presence of 18S ribosomal RNA
- minimum copy numbers of control gene mRNA transcripts, determined using quantitative gene expression techniques, as follows:
 - β-actin (amplicon length 295 bp) > 3,800 copies/100ng total RNA
 - GAPDH (amplicon length 71 bp) > 10,000 copies /100ng total RNA
- no DNA contamination

3.2 Reverse transcription coupled quantitative real-time PCR (qRT-PCR)

Asterand uses qRT-PCR for gene expression profiling and has developed an assay system that allows the determination of the abundance of more than one mRNA species in a single tube using probes that have spectrally distinct fluorophores. This assay is used routinely to measure expression of a target gene together with the expression of the ubiquitously expressed gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is used to confirm successful amplification.

3.2.1 Design of primers and probes for gene expression analysis

Primer-probe sets are designed to yield small amplicons, generally less than 150 base pairs, of the target sequence. The primer and probe sequences are subjected to homology searches against the GenBank database to confirm that they are specific for the target sequence.

3.2.2 Reverse Transcription

Total RNA is annealed to reverse primers for the target and GAPDH mRNA transcripts in an appropriate buffer, with the samples being heated to 72°C (to remove secondary structure), and then cooled to 55°C (to anneal the primers). MuLV reverse transcriptase and nucleotides is then added and the reaction mixes incubated for 30 minutes at 37°C to allow cDNA synthesis to occur. The sample is then heated to 90°C for 5 minutes to denature the reverse transcriptase.

3.2.3 qPCR

qPCR is used to simultaneously determine the expression of the target and GAPDH transcripts using cDNA prepared from total RNA. Forward and reverse primers and probes for the target and GAPDH transcripts are added to the reaction mix along with nucleotides, buffer and AmpliTaq Gold™ Taq polymerase. The PCR conditions are: 94°C for 12 minutes (enzyme activation step), followed by 40 cycles of 94°C for 15 seconds (denaturing step) and 60°C for 30 seconds (to anneal and extend).

3.3 Data analysis and Pass / Fail criteria

PCR amplification curves are analysed to yield Ct values (the fractional cycle number at which a PCR product is first detected) and these values are used to determine the starting mRNA copy number of both target and GAPDH genes by interpolation from Asterand's global standard curve (see Appendix 2).

Successful reverse transcriptions and PCRs are confirmed by co-amplification of GAPDH mRNA using a proprietary multiplexing protocol.

The following pass/fail criteria are used for gene expression profiling:

- GAPDH mRNA for each sample must be > 10,000 copies / 100ng total RNA
- No more than one failed sample in any single tissue group of 3 samples present on the XpressWay™ Profile
- No more than 3 failed samples in total on the XpressWay™ Profile

APPENDIX 1 XpressWay™ Profile tissues

Genes are profiled across a panel of 72 tissue types representing the main organ systems. Each tissue is sourced from 3 different donors.

Tissue	Tissue
heart : left atrium	brain : dorsal raphe nucleus
heart : left ventricle	spinal cord : cervical
blood vessel : coronary	dorsal root ganglion : cervical
oesophagus	pineal gland
stomach : fundus	pituitary gland
stomach : body : whole	blood vessel : choroid plexus
stomach : antrum	blood vessel : cerebral
stomach : pyloric canal	trachea
duodenum	lung : parenchyma
jejunum	lung : primary bronchus
ileum	lung : tertiary bronchus
adipose	blood vessel : pulmonary
blood vessel : mesenteric	kidney : cortex
caecum	kidney : medulla
colon	kidney : pelvis
rectum	blood vessel : renal
gallbladder	ureter
pancreas	bladder : body
liver : parenchyma	bladder : trigone
brain : cerebellum	ovary
brain : hippocampus	fallopian tube
brain : locus coeruleus	uterus : myometrium
brain : medulla	uterus : cervix
brain : amygdala	prostate
brain : caudate	vas deferens
brain : hypothalamus - anterior	testis
brain : hypothalamus - posterior	spleen : parenchyma
brain : cortex : cingulate - anterior	blood : mononuclear cells
brain : cortex : cingulate - posterior	lymph gland : tonsil
brain : cortex : frontal - lateral	skeletal muscle
brain : cortex : frontal - medial	skin
brain : cortex : occipital	adrenal gland
brain : cortex : parietal	thyroid gland
brain : cortex : temporal	umbilical cord
brain : nucleus accumbens	placenta
brain : substantia nigra	breast

APPENDIX 2

Construction of the Global Standard Curve

The criteria for successful primer-probe set design are extremely rigorous, and it has been our experience that the efficiency of amplification by PCR with sets that conform is very high. We have prepared individual standard curves for 81 genes and found it feasible to generate a global standard curve (GSC) by combining the data for these genes (Fig.1). The GSC correlates the number of PCR cycles required to reach threshold (Ct) with the starting mRNA copy number (Cn), and is used to quantify all target mRNA copy numbers.

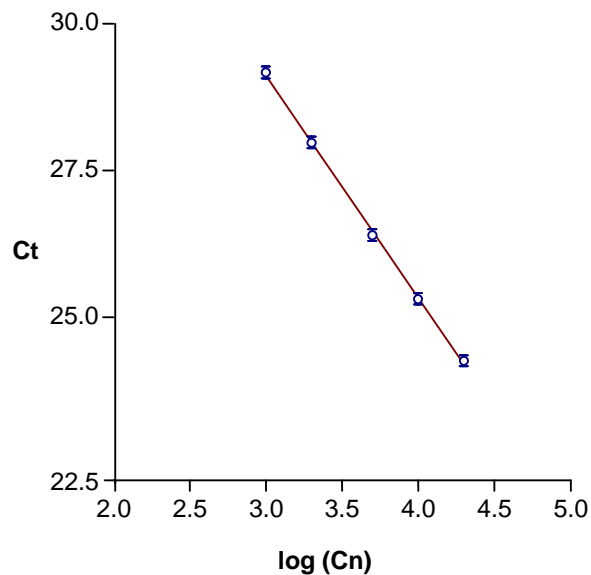


Fig. 1 Regression analysis of global standard curve data showing the mean Ct (\pm SEM) and regression fit. The relationship between log copy number (Cn) and threshold cycle (Ct) is linear. Results from 150 individual standard curves performed for 81 targets were pooled to construct the global standard curve (GSC). Linear regression analysis of the 1476 data points yielded best-fit parameters of slope of -3.623 and ordinate intercept of 40.007 .

The GSC covers a copy number range of 1000 to 20000. For some gene transcripts this has been extended to cover a wider range (50-250,000 copies). For the transcripts that have been investigated, similar results to the GSC have been obtained and a representative standard curve is shown in Fig.2. This provides confidence that extrapolation of copy number from the GSC using Ct values outside the range of the GSC tested is valid.

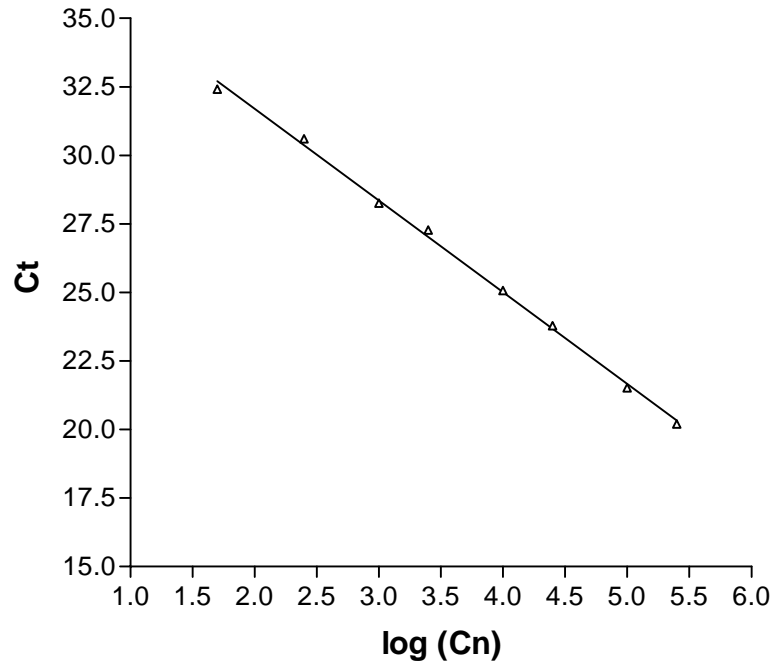


Fig. 2 Regression analysis of extended standard curve data showing the mean Ct and regression fit. Linear regression yielded best-fit parameters of slope of -3.35 and ordinate intercept of 38.4 .