

Bring in the marines!

Removal of contaminating DNA by marine
enzymes in RT-PCR

Morten Elde, Olav Lanes, Dag R Gjellesvik
Biotec Pharmacon ASA, Tromsø, Norway

Why Marine enzymes?

- Marine organisms in the northern sea are adapted to the cold environment
 - The enzymes must also be adapted
- Particular features by cold-adapted enzymes:
 - ✓ Higher activity at low temperature
 - ✓ Easy *inactivation* by moderate raise in temperature
 - ✓ Provides a very useful "off-button"

Contaminating DNA in RT-PCR

- Contaminating DNA often a problem in PCR and RT-PCR
- Previous PCR products (carry-over contaminants) or genomic DNA (RT-PCR)
- Leads to inaccurate or false positive results
- Different methods to remove contaminating DNA
 - Uracil DNA Glycosylase method (AmpErase™)
 - DNaseI
- Two new marine enzymes suitable for removal of contaminating DNA

Use of Uracil-DNA glycosylase (UNG) in carry-over prevention

- Principle: PCR product *chemically different* from template
 - dTTP is replaced by dUTP in all PCR reaction mixes
 - Uracil is incorporated instead of thymine
 - ⇒ **all PCR products contain U**
 - Uracil-DNA glycosylase (UNG) will remove U from DNA
 - PCR protocol start with UNG treatment
 - Template DNA contains T, will not degrade
 - ⇒ **NO carry over PCR contaminants are amplified**

Problems with the UNG method

- Real-time PCR/RT-PCR products are usually small with reduced number of uracils and by that harder to degrade sufficiently
- UNGs available are not suitable for carry-over prevention in RT-PCR
 - Will degrade cDNA during RT step
- Most UNGs can be reactivated

codUNG

- Uracil DNA glycosylase from the cold-adapted atlantic cod (*Gadus morhua*) was characterized
- Active at low temperatures
- Heat labile

UNG in RT-PCR

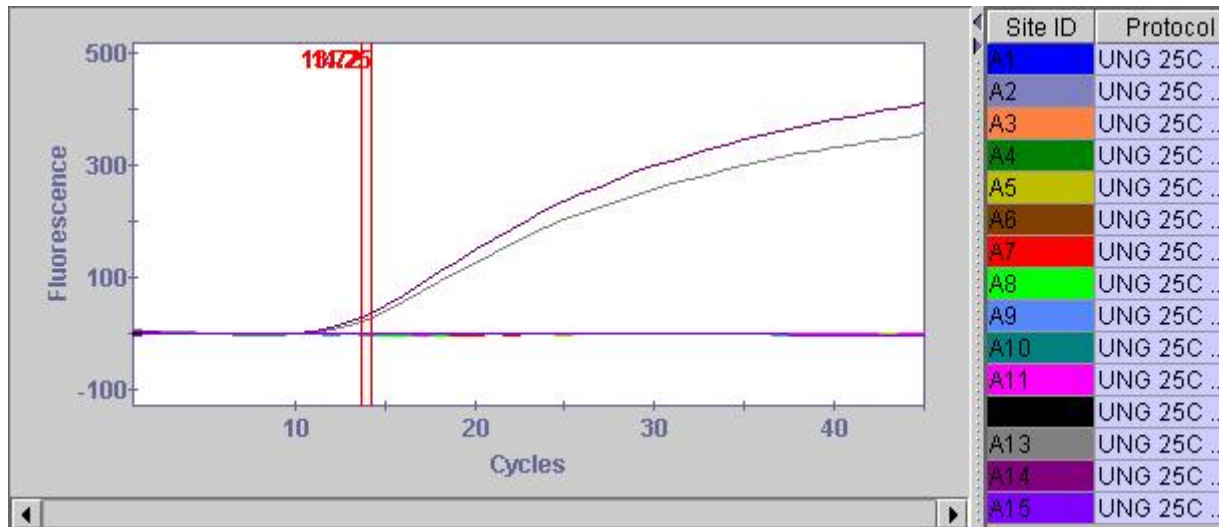
- Must be sufficiently active at low temperatures for removal of contaminating DNA
- Must be easily inactivated at reverse transcription temperature (50°C) to prevent cDNA cleavage
- Should be irreversibly inactivated if downstream analysis of PCR product necessary

Removal of contaminating DNA by codUNG

- One-step RT-PCR (close-tube)
 - Added amplicon DNA to contaminate a RT-PCR reaction (no RNA!)
 - Preincubation step with codUNG at 25/30°C, 5 min
 - Reverse transcription 50°C, 30 min
 - PCR

Results

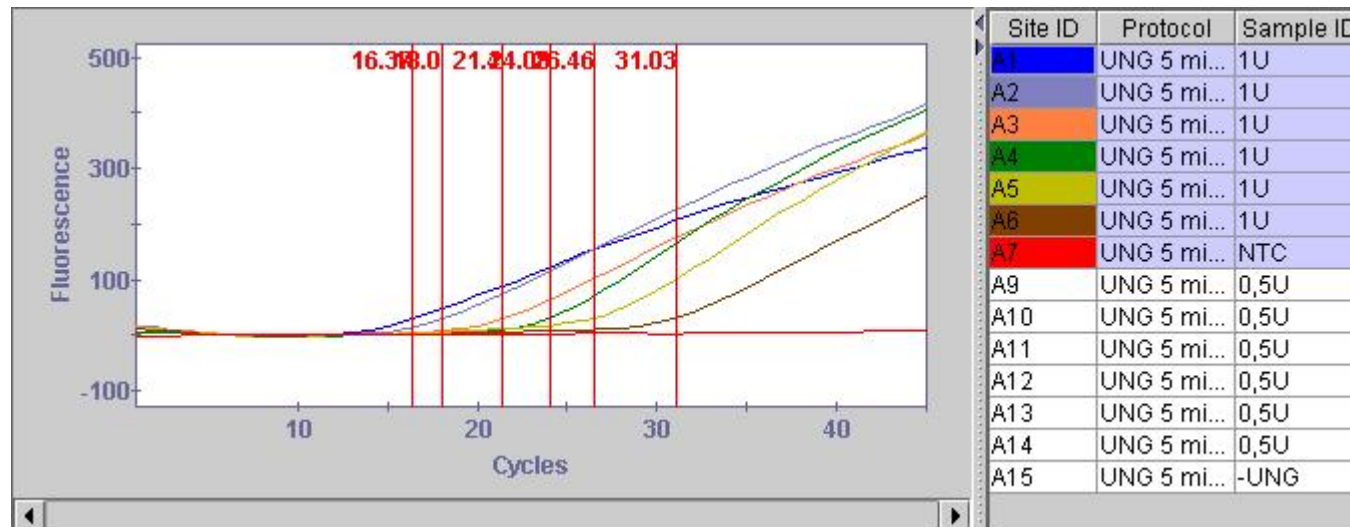
- Two controls no UNG,
- 12 parallels with 2 000 000 copies of amplicon DNA and 0.06 U codUNG



- Preincubation with a little as 0.06 units of codUNG at 25°C for 5 min is sufficient to remove vast amount of contaminating carry-over DNA

Removal with BMTU (Roche)

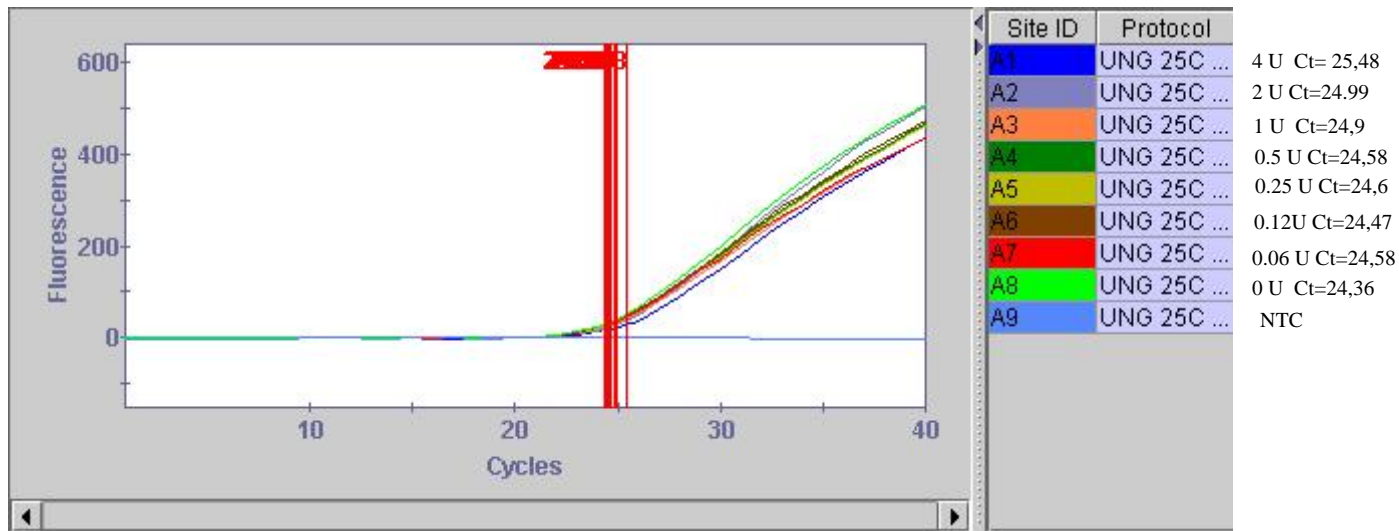
- One-step RT-PCR procedure
- 10^3 - 10^8 copies of contaminating amplicon DNA
- 1 U BMTU



Effect of codUNG on RT-PCR sensitivity

- Real-time one-step RT-PCR with 1ng human total RNA
- Different amount of codUNG
- Preincubation at 25°C for 5 min
- RT at 50°C
- PCR

Effect of codUNG on RT-PCR sensitivity



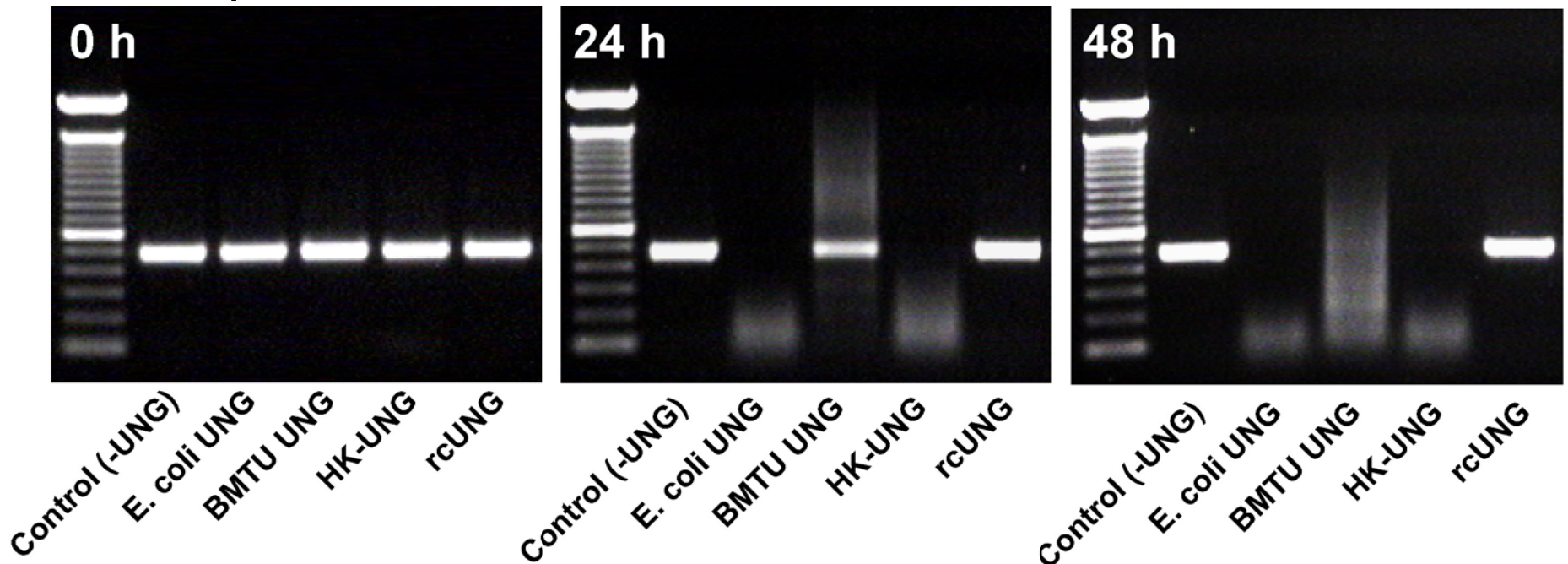
- codUNG does not significantly affect sensitivity of RT-PCR

Is the codUNG irreversibly heat-inactivated?

- If the RT-PCR product is going to be used for further analysis such as cloning, sequencing or genotyping it is important that the UNG enzyme is *irreversibly* inactivated

Inactivation/reactivation of different UNGs

- Integrity of Uracil containing PCR products at room temperature



- Only codUNG is irreversibly inactivated!

- The uracil/UNG method is not always the answer to your contamination problems in RT-PCR
 - Genomic DNA in RNA prep is not removed by UNG and can give erroneous results in real time/RT-PCR
 - Reverse transcription at a lower temperature (37°C)
 - Have to use a polymerase with proofreading
 - You don't want to pay licence-fee to Invitrogen...

- Another marine enzyme available!

Shrimp DNase

- Shrimp DNase is recombinantly expressed and purified from *Pichia pastoris*
 - Heat labile
 - 15 min at 65°C is enough to inactivate the enzyme
 - Endonuclease
 - Highly selective towards *double-stranded* DNA
 - leaves ssDNA/primers intact
 - Very low activity towards RNA compared to other commercially available DNaseI

Removal of contaminating DNA before PCR

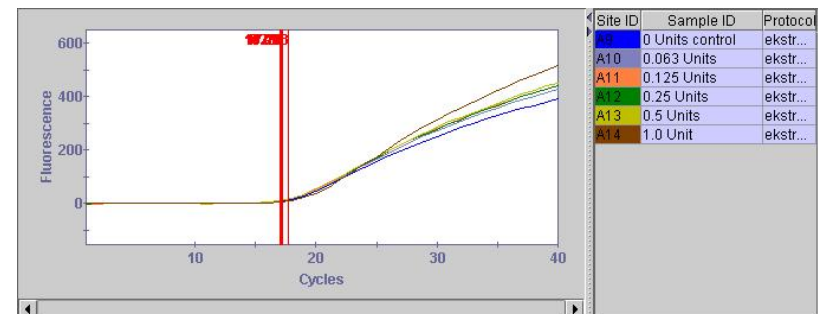
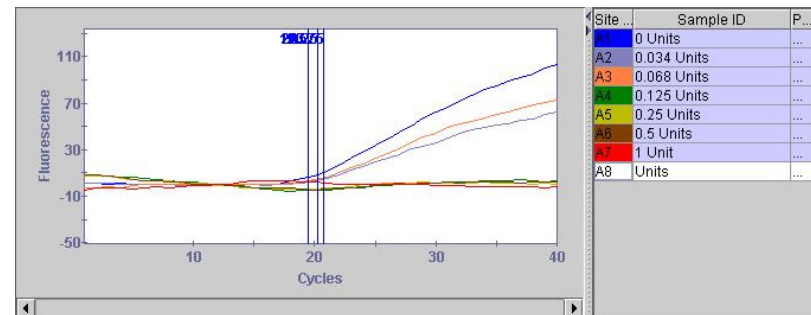
- Nuclease pretreatment of PCR reaction mix **without template**; 15min 37 °C
- Inactivate nuclease; 15min 70°C
- Add template
- Proceed with PCR

Shrimp nuclease in RT-PCR

- One-step RT-PCR with nuclease treatment
 - 42°C 15 min, Reverse Transcription of RNA/Digestion of contaminating DNA
 - 95°C 10 min. Inactivation of RT and nuclease
 - PCR
- Closed-tube protocol!

Results

- PCR product as contaminant
- Different amounts of nuclease
- Minimum amount to remove DNA: 0.125 Units
- Maximum amount without negative effect on RT-PCR: 0.5 Units
- Working window: 0.1 - 0.5 Units
- cDNA degraded to a small extent
- *User-specific optimisation is still advisable*



Conclusions

- Two marine enzymes available for removal of contaminating DNA before RT-PCR
- CodUNG
 - Removes contaminating carry-over DNA at low temperatures
 - Is easily inactivated during reverse transcription leaving cDNA intact
 - Is irreversibly inactivated
- Shrimp DNase
 - Double-strand specific nuclease removing genomic DNA from RNA preps
 - Heat labile
 - RNase free

Where can I get it?

