Transgenic Res (2011) 20:429–433
DOI 10.1007/s11248-010-9413-6

TECHNICAL REPORT

Identification of germline competent chimaeras by copulatory plug genotyping

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Received: 15 February 2010 / Accepted: 26 May 2010 / Published online: 12 June 2010

Abstract The increasing use of ES cell lines from strains other than 129, in particular C57BL/6, has greatly reduced the time taken to generate gene knockouts on a defined genetic background. Generally, C57BL/6 ES cell lines transmit less efficiently through the germline than 129 lines; consequently the burden on animal husbandry at this stage is increased. Genotyping sperm from chimaeric males may be used to identify mice which are transmitting the manipulated allele, however it requires that the mice be culled and the sperm used for IVF. Here we describe a quick and reliable method for genotyping copulatory plugs. Males which produce a positive result can then be naturally mated. Thus far we have observed a perfect correlation between copulatory plug genotype and germline transmission, accompanied by considerable savings in mouse numbers and resources.

Keywords ES cells · Germline transmission · Chimaera · C57BL/6 · Copulatory plug · Gene targeting

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Introduction

Genome manipulation via ES cells has been a firmly established technique for many years now, however until recently the only reliable ES cell lines available derived from 129 strain mice. Since phenotypic work is increasingly performed in strains other than 129, predominantly C57BL/6, this constraint makes a time-consuming backcrossing step obligatory in many cases. Recently this gap has been substantially closed by the derivation and successful targeting and germline transmission of ES cell lines from defined strains, most notably C57BL/6 (Seong et al. 2004). Despite these advancements the germline competence of C57BL/6 ES cell lines remains significantly lower than 129 derived lines (Hansen et al. 2008) and so obtaining heterozygous pups containing the targeted allele requires larger numbers of chimaeras and matings than for 129 ES cell lines. Additionally, the correlation that is usually observed between extent of coat colour chimaerism and likelihood of germline transmission does not seem to hold for C57BL/6 derived chimaeras (Seong et al. 2004). This is therefore not an optimal method for chimaera selection. This shortcoming may be addressed by genotyping sperm from male chimaeras (Katoh et al. 2005); however it requires that the mouse be culled and subsequent fertilisations be performed by IVF. We reasoned that we could circumvent these complications by genotyping the copulatory plug which is formed through the denaturation of proteins in seminal

fluid during mating and contains substantial amounts of sperm.

Here we describe a straightforward procedure optimised for procuring and genotyping copulatory plugs, and demonstrate perfect correlation between detection of the target allele and its germline transmission. To our knowledge this is the first report of copulatory plugs being used to obtain information to direct breeding during gene targeting by homologous recombination.

Methods, results, and discussion

All animal procedures were reviewed and approved by the GlaxoSmithKline Animal Care and Use Committee, and were performed in accredited facilities in accordance with institutional guidelines and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

ES cell lines, chimaera generation and breeding procedures

Chimaeras were generated by microinjection of gene targeted ES cells derived from C57BL/6J: JAX® or BALB/cAnNCrl mice (GSK derived ES cells) into BALB/cOlaHsd or C57BL/6J: JAX® host blastocyst stage embryos. At sexual maturity (~7 weeks of age) male chimaeras were mated as trios with two C57BL/6J: JAX® or BALB/cAnNCrl female mice. The female mice were observed daily for the presence of a copulatory plug. Once found the plugged female was removed and culled, plug harvested and placed into an Eppendorf tube, then immediately frozen at −80°C. Plugs were stored at −80°C until genotyping was carried out. The remaining female was left paired with the male chimaera, generating two litters (~10–16 pups) to observe germline transmission by coat colour and subsequent genotyping of animals.

To minimise animal usage we initially used retired overweight CD1 females from pseudopregnant matings to obtain chimaera copulatory plugs. This proved unsuccessful as frequently, even after 2 weeks of mating, copulatory plugs were not obtained from all of the chimaeras. We therefore recommend that females of optimum breeding age and strain are used.

Sperm cryopreservation and tissue harvest

Male chimeras which had been used for germline transmission breeding were culled by cervical dislocation.

Sperm was harvested and cryopreserved as described in Nagy et al. (2003). Briefly, cauda epididymides were harvested from freshly sacrificed animals and placed into cryprotective agent (CPA—consisting of 18% Raffinose and 3% Skim Milk). Tears were made in the tissue using a 30 gauge needle and left for 10 min to release the sperm. Epididymal tissue was removed, the sperm/CPA solution was then mixed and 100 µl aliquots transferred into cryovials. These were cooled in liquid nitrogen vapour phase for 10 min then plunged directly into the liquid nitrogen.

To prepare sperm for genotyping, a vial of sperm/CPA was thawed rapidly at 37°C. The contents were centrifuged at 3,000 rpm for 4 min in a microfuge. The supernatant was removed and discarded and the remaining sperm pellet was snap frozen and stored at −80°C until genotyping was carried out.

DNA extraction

Distal tail-tips (< 5 mm in length), sperm and copulatory plugs were digested in 250 µl Nucleon reagent M1 (Genprobe) containing 100 µg/ml Proteinase K overnight at 55°C. On the following day, 100 µl of Nucleon reagent M2 plus 75 µl Nucleon resin was added and vigorously mixed. Solid material was pelleted by centrifugation at 13,000 rpm for 5 min and 50 µl supernatant transferred to an equilibrated Microspin 400HS minicolumn (GE Healthcare) and centrifuged at 3,000 rpm for 1 min. The resulting eluate was then heated to 99°C for 10 min to ensure inactivation of residual Proteinase K. This simple and rapid DNA clean-up procedure provides an excellent substrate for PCR.

Determination of genotypes

Genotypes of all tissues were assayed by realtime PCR performed in 15 µl reactions on an ABI 7900HT system using ABI Taqman mastermix with primers added to a final concentration of 750 nM, probe to 100 nM, and approximately 20 ng template DNA (typically 1 µl of the above preparation). Cycling parameters were default ABI Taqman from the 384 well plate template. Primers

were designed to detect the neomycin resistance gene (*neo*) which is common to all of the lines assayed in this study. Realtime PCR assays were performed in quadruplicate and normalised to a wild type genomic control run as a duplex reaction with a JOE-labelled control primer/probe set (Table 1). Graphs are expressed as a normalised ratio of *neo*:WT signal. PCR assays for conventional gel electrophoresis were performed using Qiagen Multiplex Mastermix with targeted allele-specific primers added to a final concentration of 200 nM and template DNA as above, in a 20 µl reaction. We used the following touchdown cycling protocol which is extremely robust in our hands: 95°C 10 min for 1 cycle; 10 cycles of 95°C 15 s, 65°C (minus 1°/cycle) 15 s, 72°C 30 s; 25 cycles of 95°C 15 s, 55°C 15 s, 72°C 30 s. PCR products were run on a 2% agarose gel stained with SYBRsafe (Invitrogen), and visualised by blue light transillumination.

Sperm *neo* signal correlates with ES cell germline transmission

We first explored the correlation between sperm genotype and germline transmission. To this end we genotyped sperm samples from 7 mice which had already produced litters of known genotype (Fig. 1).

In line with our expectations, sperm from the germline competent chimaera produced a high n*eo* signal that was easily distinguishable from the non-transmitting mice. Encouraged by this result we proceeded to investigate the feasibility of genotyping copulatory plugs.

Copulatory plug *neo* signal correlates with germline transmission of the targeted allele

From 54 copulatory plugs we identified 13 which were positive for *neo* (Fig. 2). All of these donor chimaeras subsequently gave rise to ES cell clone derived offspring containing the targeted allele (Table 2). Contrary to our expectations we did not

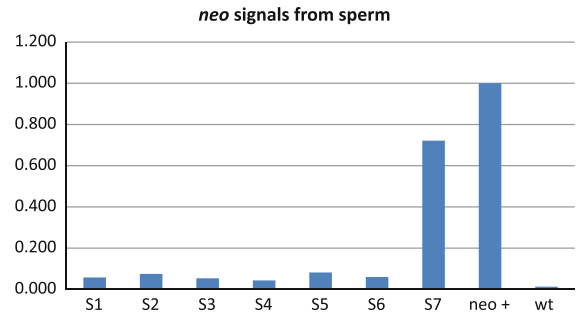

Fig. 1 Correlation between sperm *neo* genotype and germline transmission. *Bars* represent the *neo* signal normalised to a heterozygous control sample from mice 1–7. Only mouse 7 produced germline transmission

observe a reproducible correlation between *neo* signal strength and the rate of germline transmission although sample numbers are too low to draw any firm conclusion in this regard. With the exception of chimaera 11 which sired 34 pups with only one *neo* positive, efficient germline transmission of the targeted allele was attained regardless of the relative *neo* signal. The reason for this lack of correlation is unclear, however it is well known within the field that ES cell derived sperms sometimes mature at a different rate than those of the host and so it is conceivable that ejaculates produced at different times might differ markedly in their ES cell derived sperm component. We made no attempt to measure this in these experiments.

Three chimaeras produced *neo* negative plugs yet generated ES cell clone derived progeny by coat colour. In all cases the pups were *neo* negative and so, as predicted, the targeted allele was not transmitting through the germline. No ES cell clone derived progeny were produced by any of the remaining *neo* negative plug chimaeras.

Mating plug genotyping by conventional PCR

Finally, we considered whether conventional PCR would be sufficiently sensitive to detect the presence

Table 1 Realtime PCR primers used in this study

Neo326F	ACCTTGCTCCTGCCGAGAAAGTAT
Neo428R	CGATGTTTCGCTTGGTGGTCGAAT
Neo366_FAM	6FAM-AATGCGGCGGCTGCATACGCTTGAT-Iowa Black
KIAA-F	TCTCATGGGCCTGACTTTCCCTTT
KIAA-R	ACCCTGCACACAAACACATTCACC
KIAA-P	JOE-AAAGTCCTTGGTGACTCAGGCCTCG-Iowa Black

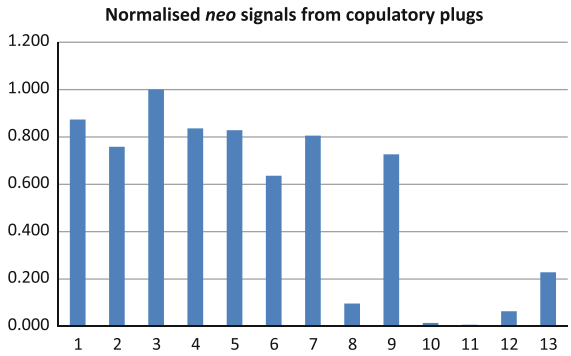

Fig. 2 Correlation between *neo* positive signal and germline transmission. *Bars* represent the relative *neo* signal detected in the sample (arbitrary scale). Chimaeras 1–9 were derived from BALB/c ES cells, 10–13 from C57BL/6

of transgene containing sperm in the copulatory plugs. Using a subset of our existing plug samples, we repeated the assays using both realtime PCR and an allele-specific primer set and compared the outcome (Fig. 3).

The four plugs which returned the highest signal in the realtime PCR assay also produced clear positive signals by conventional PCR. All of the chimaeras that these plugs were derived from went onto produce ES-cell derived progeny. One very weak realtime PCR positive failed to produce an allele-specific conventional PCR product, however this mouse had assayed neo negative in the previous experiment, and had failed to produce any ES cell derived pups, and so we believe that it was an artifactual false positive in this experiment.

Conclusion

We have devised a simple and rapid copulatory plug genotyping procedure that reliably predicts the germline competence of chimaeras. The level of correlation between signal intensity and rate of germline transmission was somewhat variable, importantly though the presence of a positive signal was always predictive of successful germline transmission. The method that we have described works well using realtime PCR amplifying a genetic marker common to the majority of targeting vectors and also worked robustly by conventional PCR with a primer pair specific to the targeted allele. It is of course straightforward to apply this method to any transgene of interest.

The GSK derived BALB/c ES cells transmitted very readily through the germline; in this situation copulatory plug genotyping conferred no significant advantage, although any saving in animal usage should be considered.

For C57BL/6 ES cells though, where germline transmission rates are poor, the advantages are clear. Consistent with the observations of others (Ware et al. 2003) we suggest that three to four independent clones derived from C57BL/6 ES cells for each gene targeting project, are required to efficiently attain germline transmission. It has been our experience with C57BL/6 ES cells that coat colour contribution is a poor indicator of germline transmission rate (most of the plug negative chimaeras that we assayed had a very high ES cell coat colour contribution—data not shown). We therefore recommend that all chimaeras be used for the initial copulatory plug screen.

The breeding scheme that we used was designed to test our hypothesis and to obtain sufficient data to build confidence in the outcome. This enabled us to obtain copulatory plugs efficiently, usually within 4 days of initial mating. For future experiments we propose that chimeras are only paired with one female to obtain a copulatory plug for genotyping, this will minimise animal usage and negate the need to generate unnecessary litters. It is not unusual for ES cell-mouse chimeras to produce one or more litters without germline transmission. Only in later litters are germline pups produced by these chimeras. This has not been our experience with our C57BL/6 and BALB/c ES cell lines, so we would normally cull plug negative chimaeras after a single assay, however where delayed germline transmission has been observed it may be prudent to retain chimaeras and re-assay after a suitable time has elapsed. Once allele positive plugs

Table 2 Percentage ES cell contribution to chimaera coat colour and percent ES cell germline transmission (GLT) for the chimaeras described in Fig. 2

	1	2	3	4	5	6	7	8	9	10	11	12	13
Chimaera Coat Colour	>70%	>70%	>70%	>70%	>70%	>70%	>70%	>70%	>70%	40–70%	<40%	40–70%	>70%
Coat Colour GLT	100%	100%	100%	100%	100%	100%	100%	67%	100%	100%	5%	100%	100%

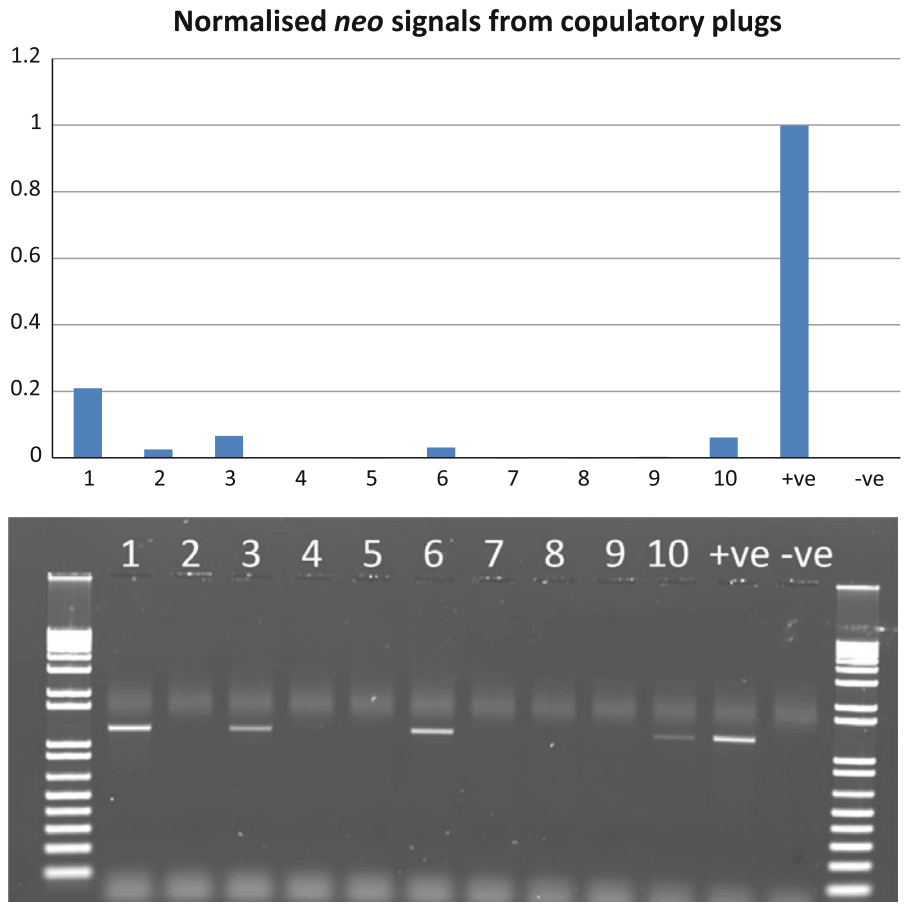

Fig. 3 Comparison between real-time (*upper panel*) and conventional (*lower panel*) PCR for copulatory plug genotyping

are identified, breeding can commence with only those chimaeras until the requisite number of heterozygous progeny have been obtained.

The implications for animal welfare are profound. Taking the small number of mice utilised in this study as an example, the 41 non-transmitting chimaeras bred under our conventional germline transmission breeding protocol, which had already been optimised (4 litters sired per chimaera) would have sired around 820 negative pups and used an additional 41 females; clearly these would not have been produced had we elected only to breed from plug *neo* positive chimaeras. It is obvious that a reliable method for pre-selecting the best chimaeras will save many thousands of mice, particularly viewed in the context of the large-scale public gene knockout consortia.

Acknowledgments We would like to thank GSK Laboratory Animal Sciences, especially Will Traylen for mouse husbandry and plug harvesting and Ellen Shapland of GSK Molecular Discovery Research for assistance with chimaera generation.

References

Hansen GM, Markesich DC, Burnett MB, Zhu Q, Dionne KM, Richter LJ, Finnell RH, Sands AT, Zambrowicz BP, Abuin A (2008) Large-scale gene trapping in C57BL/6 N mouse embryonic stem cells. Genome Res 18:1670–1679. doi:10.1101/gr.078352.108

Katoh H, Yoshino S, Inui Y, Honda S, Takabayashi S (2005) Microsatellite genotyping for genetic quality testing using sperm cells in the mouse. Exp Anim 54:373–376. doi:10.1538/expanim.54.373

Nagy A, Gertsenstein M, Vintersten K, Behringer R (2003) Manipulating the mouse embryo, 3rd edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour

Seong E, Saunders TL, Stewart CL, Burmeister M (2004) To knockout in 129 or in C57BL/6: that is the question. Trends Genet 20:59–62. doi:10.1016/j.tig.2003.12.006

Ware CB, Siverts LN, Nelson AM, Morton JF, Ladiges WC (2003) Utility of a C57BL/6 ES line versus 129 ES lines for targeted mutations in mice. Transgenic Res 12:743–746. doi:10.1023/B:TRAG.0000005246.35812.c8